

ASM CONFERENCE ON
MACROMOLECULAR TRANSPORT
ACROSS CELLULAR MEMBRANES

May 31 – June 4, 2000
Savannah, Georgia



**AMERICAN
SOCIETY FOR
MICROBIOLOGY**

AD _____

Award Number: DAMD17-00-1-0592

TITLE: Conference "Macromolecular Transport Across Cellular Membranes"

PRINCIPAL INVESTIGATOR: Robert Jensen, Ph.D.

CONTRACTING ORGANIZATION: American Society for Microbiology
Washington, DC 20036

REPORT DATE: September 2000

TYPE OF REPORT: Final Proceedings

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution unlimited

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20010102 146

DTIC QUALITY INDEXED 4

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

Management and Budget, Project Number: Object (070-0100), Washington, DC 20000			
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Final Proceedings (1 May 00 - 30 Jul 00)	
4. TITLE AND SUBTITLE Conference "Macromolecular Transport Across Cellular Membranes"		5. FUNDING NUMBERS DAMD17-00-1-0592	
6. AUTHOR(S) Robert Jensen, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) American Society for Microbiology Washington, DC 20036		8. PERFORMING ORGANIZATION REPORT NUMBER	
E-MAIL: rjensen@jhmi.edu			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; Distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words)			
See Report			
14. SUBJECT TERMS		15. NUMBER OF PAGES 43	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

ASM CONFERENCE ON

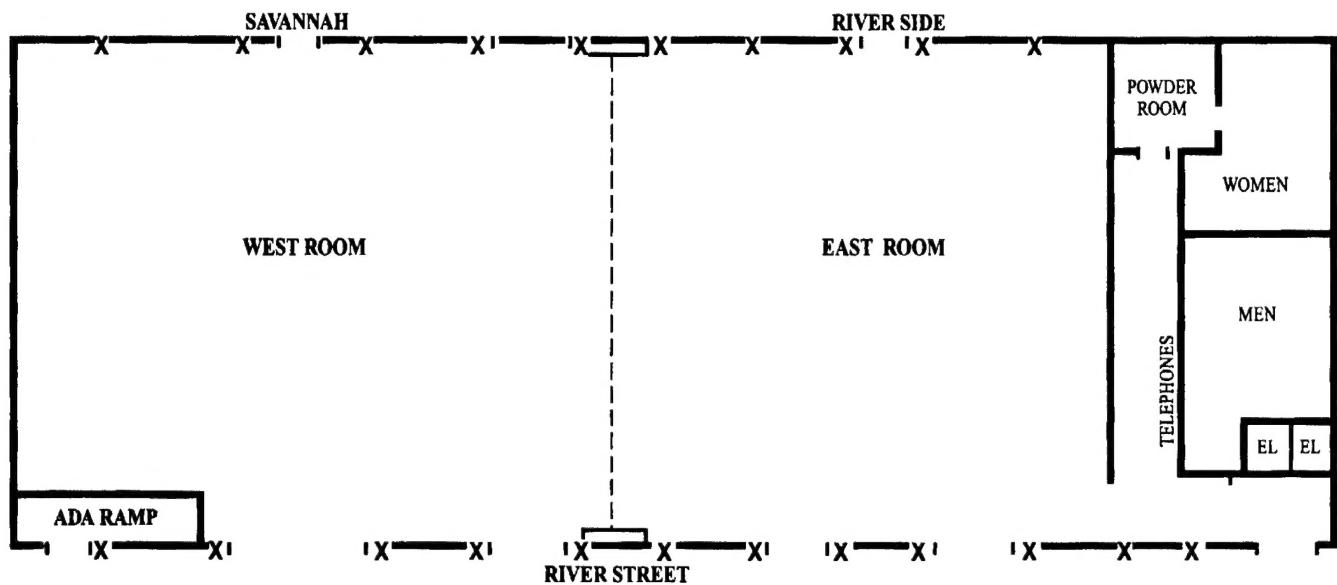
MACROMOLECULAR TRANSPORT ACROSS CELLULAR MEMBRANES

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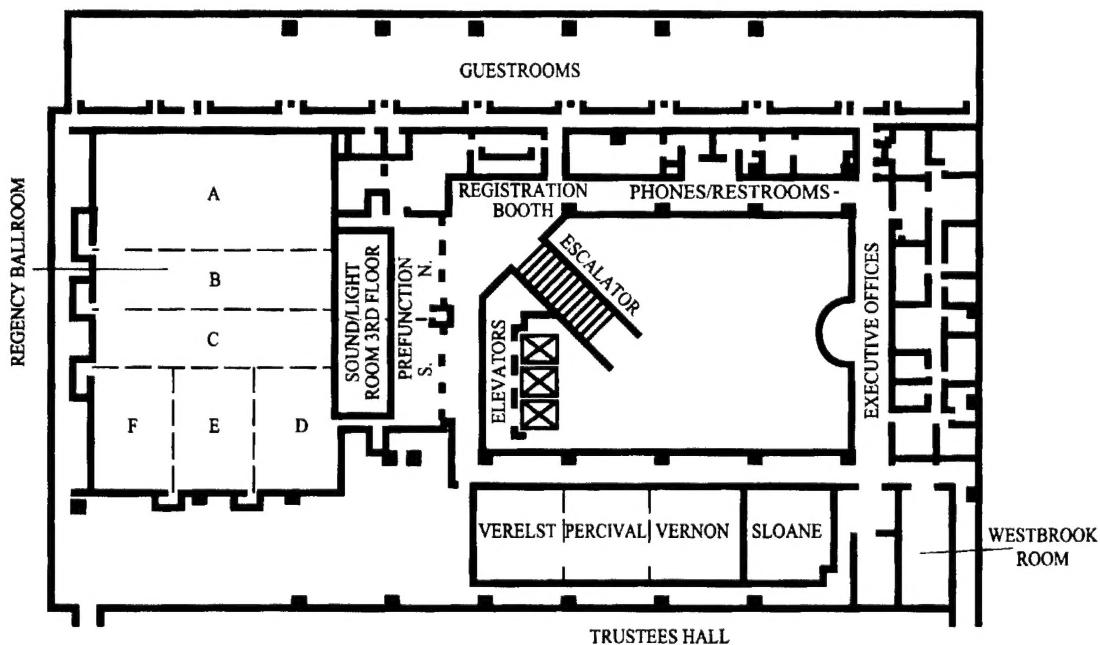
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MAP OF THE HYATT REGENCY SAVANNAH

HARBORSIDE CENTER



SECOND FLOOR



GENERAL INFORMATION

SCIENTIFIC PROGRAM ORGANIZERS

Rob Jensen
Department of Cell Biology and Anatomy
Johns Hopkins School of Medicine

Art Johnson
Department of Medical Biochemistry and Genetics
Texas A&M University System Health Sciences Center

REGISTRATION

During the scientific sessions the conference registration desk will be located outside the Regency Ballroom and staff will be available to assist you with any requests or questions during session hours. Certificates of attendance are available from the ASM staff at this desk.

GENERAL SESSIONS

All general (oral presentation) sessions will be held in the Regency Ballroom. A name badge is required for all sessions.

POSTER SESSIONS

Poster sessions will be held in the Harborside Center at the Hyatt. Posters will be available for informal viewing throughout the conference. Abstract numbers correspond to poster board numbers.

Presenters should consult the index at the back of this book to find their assigned board number.

CONFERENCE MEALS

As shown on the following program schedule, breakfasts, a banquet and coffee breaks are included with registration for conference participants. Lunches and dinners are not included, but the schedule allows ample time to have meals on your own. Breakfasts and the banquet will be in the Harborside Center.

ACKNOWLEDGMENTS

The American Society for Microbiology and the Scientific Program Organizers of the conference gratefully acknowledge the contributions of the following for their support:

Promega Corporation
Merck Research Laboratories
Beckman Coulter

SCIENTIFIC PROGRAM

WEDNESDAY, MAY 31, 2000

12:00 – 9:00 pm: **Registration Open**

7:30 – 7:40 pm: **Welcome and Information**

7:40 – 9:15 pm: **Session I: How are Macromolecules Selected and Targeted for Translocation?**
Session Chair: Koreaki Ito, Kyoto University, Kyoto, Japan

7:40 – 7:45 pm: **Remarks by Session Chair**

7:45 – 8:15 pm: **Molecular Recognition of Newly Synthesized Peroxisomal Proteins**
Steve Gould, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

8:15 – 8:45 pm: **Protein and Peptide Translocation from the ER Lumen to the Cytosol**
Karin Römisch, University of Cambridge, Cambridge, United Kingdom.

8:45 – 9:15 pm: **GTP-Dependence of Signal Recognition Particle Binding to Ribosome-bound Signal Sequences**
Art Johnson, Texas A&M University System Health Sciences Center, College Station, TX, USA.

9:15– 11:00 pm: **Reception**

THURSDAY, JUNE 1, 2000

7:00 - 8:30 am: **Breakfast**

8:30 - 11:45 am: **Session II: How are Macromolecules Selected and Targeted for Translocation? (continued)**
Session Chair: Reid Gilmore, University of Massachusetts Medical School, Worcester, MA, USA.

8:30 – 8:35 am:	Remarks by Session Chair
8:35 – 9:05 am:	A Family of Receptors for Targeting Preproteins to Chloroplasts Danny Schnell, Rutgers University, Newark, NJ, USA.
9:05 – 9:25 am:	Characterization and Subcellular Localization of the Peroxisomal Membrane Protein, PMP50 Christopher Sans, University of Western Ontario, London, Ontario, Canada.
9:25 – 9:55 am:	The Physiological Basis for the Conservation of the Signal Recognition Particle Targeting Pathway in E.Coli Harris Bernstein, National Institutes of Health, Bethesda, MD, USA.
9:55 – 10:25 am:	Coffee Break
10:25 – 10:55 am:	Mechanism of Protein Import into the Peroxisomal Matrix and Membrane William Snyder, University of California, San Diego, CA, USA.
10:55 – 11:15 am:	A Family of Receptors for Targeting Preproteins to Chloroplasts Georg Koch, Universität Freiburg, Freiburg Baden-Wurtemberg, Germany.
11:15 – 11:45 am:	Retrotranslocation and Degradation of Integral Membrane Proteins Ron Kopito, Stanford University, Stanford, CA, USA.
11:45 am – 2:00 pm:	Lunch Break
2:00 – 4:45 pm:	Session III: What Components Comprise the Translocating Sites or Translocons? How are Proteins Arranged in Translocating Sites? How does the Translocon Interact with Macromolecules in Transit? <i>Session Chair: Nikolaus Pfanner, Universität Freiburg, Freiburg Baden-Wurtemberg, Germany.</i>
2:00 – 2:05 pm:	Remarks by Session Chair
2:05 – 2:35 pm:	Insertion of Polytopic Proteins into the Mitochondrial Inner Membrane Rob Jensen, Johns Hopkins School of Medicine, Baltimore, MD, USA.
2:35 – 2:55 pm:	<i>Yidc, the E. coli Homologue of Mitochondrial Oxalp is a Component of the Sec Translocase</i> Pier Scotti, Biocentrum Amsterdam, Amsterdam, The Netherlands.
2:55 – 3:25 pm:	Coffee Break

3:25 – 3:55 pm:	Characterizing Functional Domains of Nup153, a Central Player in the Nucleocytoplasmic Translocon Katie Ullman, University of Utah, Salt Lake City, UT, USA.
3:55 – 4:15 pm:	The Mammalian Homolog of the Yeast Sec Complex Subunit Sec63p: Part of a Multifunctional Transport Complex Martin Jung, Universität Saarlandes, Hamburg, Germany.
4:15 – 4:45 pm:	Connections Between Nucleoporins, Inositol Signaling, and mRNA Export Susan Wente, Washington University, St. Louis, MO, USA.
4:45 - 7:00 pm:	Poster Session I (with wine/cheese) Evening Free

FRIDAY, JUNE 2, 2000

7:00 - 8:30 am:	Breakfast
8:30 – 11:45 am:	Session IV: How are Translocating Sites Assembled? <i>Session Chair: Walter Neupert, Ludwig-Maximilians-Universität München, Munich, Germany.</i>
8:30 – 8:35 am:	Remarks by Session Chairs
8:35 – 9:05 am:	The Protein Import Machinery of Yeast Mitochondria Nikolaus Pfanner, Universität Freiburg, Freiburg Baden-Wurtemberg, Germany.
9:05 – 9:25 am:	The Ins and Outs of Transmembrane Protein Folding. Membrane Protein Topogenesis from a Lipid Perspective Mikhail Bogdanov, University of Texas Medical School, Houston, TX, USA.
9:25 – 9:55 am:	The Mechanisms of SRP Receptor Assembly in Eukaryotes and <i>E. coli</i> Suggest a Novel Role in Regulating Unidirectional Protein Targeting David Andrews, McMaster University Health Science Center, Hamilton, Ontario, Canada.
9:55 – 10:25 am:	Coffee Break

10:25 – 10:55 am: **Assembly of an Oligomeric ABC Transporter**
Beth Traxler, University of Washington, Seattle, WA, USA.

10:55 – 11:15 am: **Component Organization of the Machinery for the Thylakoidal Delta pH/Tat Pathway**
Hiroki Mori, University of Florida, Gainesville, FL, USA.

11:15 – 11:45 am: **Mechanism of Proofreading by the Bacterial Preprotein Translocase**
Arnold Driessens, Groningen Biomolecular Sciences and Biotechnology Institute, Groningen, The Netherlands.

11:45 am – 7:30 pm: **Afternoon Break**

7:30 – 9:45 pm: **Session V: What are the Shapes and Structural Features of Translocating Sites? What is Known About Other Mechanisms for Transporting Macromolecules Across Membranes?**
Session Chair: Colin Robinson, University of Warwick, West Midland, United Kingdom.

7:30 – 7:35 pm: **Remarks by Session Chair**

7:35 – 8:05 pm: **Mechanisms of TOM and TIM Translocases**
Walter Neupert, Ludwig-Maximilians-Universität München, Munich, Germany.

8:05 – 8:25 pm: **Projection structure and oligomeric properties of a bacterial core protein translocase**
Ian Collinson, Harvard Medical School, Boston, MA, USA.

8:25 – 8:55 pm: **Mechanochemistry of the ABC Transporters**
Phil Thomas, University of Texas Southwestern Medical Center, Dallas, TX, USA.

8:55 – 9:15 pm: **Cell-Penetrating Peptides, a Tool for Cellular Delivery of Hydrophilic Macromolecules**
Anna Elmquist, University of Stockholm, Stockholm, Sweden.

9:15 – 9:45 pm: **Structural Basis of Presequence Recognition and Transmembrane Movement During Protein Import into Mitochondria**
Toshi Endo, Nagoya University, Nagoya, Japan.

SATURDAY, JUNE 3, 2000

7:00 - 8:30 am: **Breakfast**

8:30 – 11:55 am: **Session VI: What Powers Macromolecular Movement? Must Macromolecules be Unfolded?**
Session Chair: Steve Gould, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

8:30 – 8:35 am: **Remarks by Session Chair**

8:35 – 9:05 am: **Targeting of Proteins to the Thylakoid Membrane and Bacterial Plasma Membrane**
Colin Robinson, University of Warwick, West Midland, United Kingdom.

9:05 – 9:35 am: **The Movement of Transport Carriers across the Nuclear Pore Complex**
Mary Moore, Baylor College of Medicine, Houston, TX, USA.

9:35 – 10:05 am: **Mechanism of tRNA Import into Mitochondria**
Steve Hajduk, University of Alabama at Birmingham, Birmingham, AL, USA.

10:05 – 10:35 am: **Coffee Break**

10:35 – 11:05 am: **The Mechanism of Protein Unfolding by Mitochondria**
Andreas Matouschek, Northwestern University, Evanston, IL, USA.

11:05 – 11:25 am: **Selectivity in Sorting of Preassembled Cytosolic PTS1-Proteins in the Yeast Hansenula polymora**
Klaas Nico Faber, University of Groningen, Groningen, The Netherlands

11:25– 11:55 am: **Energetics of Protein Transport on the Thylakoid Delta-pH-Dependent Pathway**
Steve Theg, University of California, Davis, CA, USA.

11:55 am – 2:00 pm: **Lunch Break**

2:00 – 4:55 pm:	Session VII: How Do Translocon Components Interact with Each Other? How Are Translocating Sites Regulated? How Do Translocons Affect Integration of Proteins into Membranes? <i>Session Chair: Hidde Ploegh, Harvard Medical School, Boston, MA, USA.</i>
2:00 – 2:05 pm:	Remarks by Session Chair
2:05 – 2:35 pm:	The Mechanism of Ribosome Exchange on the Endoplasmic Reticulum Membrane Chris Nicchitta, Duke University Medical Center, Durham, NC, USA.
2:35 – 3:05 pm:	Subunit Interactions of the <i>E. coli</i> Protein Translocase Koreaki Ito, Kyoto University, Kyoto, Japan.
3:05 – 3:35 pm:	Import of Mitochondrial Inner Membrane Proteins Carla Koehler, University of California, Los Angeles, CA, USA.
3:35 – 4:05 pm:	Coffee Break
4:05 – 4:25 pm:	Site-Specific Phosphorylation Used as a Marker for Membrane Protein Topology and as a Timer to Estimate the Timer-Course of SRP-Dependent Protein Targeting to the Endoplasmic Reticulum Veit Goder, University of Basel, Basel, Switzerland.
4:25 – 4:55 pm:	Transfer of the Ribosome Nascent Chain Complex from the SRP to the Translocon: Regulation by Three GTPases Reid Gilmore, University of Massachusetts Medical School, Worcester, MA, USA.
6:00 - 8:00 pm:	Poster Session II (with microbrew tasting)
8:00 pm:	Banquet/ Party with DJ and Dancing

SUNDAY, JUNE 4, 2000

7:00 - 8:30 am: **Breakfast**

8:30 - 11:20 am:	Session VIII. How do Translocating Sites Work in Reverse (Retrotranslocation)? <i>Session Chair: Ron Kopito, Stanford University, Stanford, CA, USA.</i>
8:30 – 8:35 am:	Remarks by Session Chair
8:35 – 9:05 am:	Unique Chaperone Requirements Define the ER Associated Degradation (ERAD) of Soluble and Membrane Proteins Jeff Brodsky, University of Pittsburgh, Pittsburgh, PA, USA.
9:05 – 9:25 am:	A Microsomal GTPase is Required for Glycopeptide Export from Mammalian Endoplasmic Reticulum Bassam R.S. Ali, Imperial College of Science, Technology and Medicine, London, United Kingdom.
9:25 – 9:45 am:	Filamentous Phage f1 are Transported Across Membranes Through phage-Encoded Aqueous Channels Sandy Simon, Rockefeller University, New York, NY, USA.
9:45 – 10:15 am:	Coffee Break
10:15 – 10:45 am:	Co-Translational Interactions of Apoprotein B with the Ribosome and Translocon During its Assembly with Lipids in the Endoplasmic Reticulum or its Targeting to Proteasomal Degradation in the Cytosol Ed Fisher, Mount Sinai School of Medicine, New York, NY, USA.
10:45 – 11:15 am:	Dislocation of MHC Class I Proteins by CMV Products: an Update Hidde Ploegh, Harvard Medical School, Boston, MA, USA.
11:15 – 11:20 am:	Closing and Thanks

GENERAL SESSION/ INVITED PRESENTER ABSTRACTS

S1. Selective Export from the Endoplasmic Reticulum (ER) to the Cytosol

Karin Römisch, University of Cambridge, Cambridge Institute for Medical Research, Hills Road, Cambridge CB2 2XY, U.K.

In eukaryotic cells, peptides and misfolded secretory proteins are efficiently removed from the secretory pathway in order to prevent them from interfering with secretion of functional proteins and cell-to-cell communication. This 'garbage removal' occurs in the first compartment of the secretory pathway, the ER. Misfolded proteins and peptides are transported across the ER membrane to the cytosol where the proteins are degraded by proteasomes. Secretory protein import into the secretory pathway occurs through a channel in the ER membrane lined by the polytopic Sec61 protein. Using conditional mutants in SEC61 we have shown that this channel is also responsible for export of both proteins and glycopeptides from the ER. We are now trying to understand the regulation of these transport processes through the Sec61 channel, and the mechanism of targeting for export from the ER lumen to the cytosol. We found that the peptide binding site, but not the enzymatic activity of ER-luminal protein

disulfide isomerase (PDI) is required for export of a misfolded protein from the ER to the cytosol, which suggests that PDI recognizes misfolded proteins as export substrates and may be involved in the targeting process. Point mutations in SEC61 differentially affect protein export and peptide export to the cytosol, indicating that different features of the channel are important for transport of specific substrates or that different accessory proteins interact with the channel during protein and peptide export.

S2. Characterization and Subcellular Localization of the Peroxisomal Membrane Protein, PMP50

C. Sans, C. Brocard, P. Walton

University of Western Ontario, London, ON Canada

Evidence suggests that nascent vesicles of ER origin may be necessary for the formation of functional peroxisomes. The recently identified 50 kDa membrane-associated protein, termed PMP50, is the only mammalian peroxisomal protein known to be synthesized on membrane-bound polysomes. PMP50 co-sedimented with peroxisomal membranes and pulse-chase experiments indicated that it first associated with the ER and then accumulates with peroxisomes. Here we show that upon microinjection of alcohol oxidase, a yeast peroxisomal matrix protein, mammalian cells exhibit a punctate staining pattern specific for PMP50 but these vesicles do not contain catalase, a bona-fide peroxisomal marker. Database searches indicate strong homology between PMP50, the vesicular transport protein ERGIC-53, and the Galectins, a family of proteins with galactose binding signatures. Based on previous work and this sequence

similarity, we hypothesized that PMP50 may act as a shuttle between the ER and peroxisomes. Indeed, in yeast, some peroxisomal proteins were demonstrated to be glycosylated. Galectins have an affinity for -galactose sugars. To examine the binding specificity of PMP50, we have microinjected sugars into the cytosol of rat fibroblasts transfected with a full-length HA-tagged version of PMP50. We found that upon the microinjection of lactose, but not sucrose, PMP50 exhibits a cytoplasmic staining pattern, indicating that PMP50 has a stronger affinity for lactose and therefore may bind preferentially to lactose-containing molecules. In order to further study the subcellular localization of PMP50, we have constructed full length and C-terminal HA-tagged versions of PMP50 and have produced a stable cell line. We have examined the association of PMP50 with peroxisomes. Digitonin, salt and Triton X-114 phase separation experiments, followed by western blot analysis revealed that PMP50 is found almost exclusively in membrane fractions. Immunofluorescence studies showed that PMP50 exhibits a vesicular staining pattern and some, but not all of these vesicles colocalize with the peroxisomal markers PMP70 and thiolase. Transient transfections of truncated versions of PMP50-HA showed that the C-terminal end (AA107-323) of this hybrid protein binds to vesicles indicative of peroxisomes. We propose that PMP50 may be directly involved in peroxisome biogenesis by participating in the shuttling of cargo and membrane components of newly formed peroxisomes.

S3. The Physiological Basis for the Conservation of the Signal Recognition Particle Targeting Pathway in *E. coli*

H. D. Bernstein, J. Hyndman

National Institutes of Health, Bethesda, MD

The *E. coli* signal recognition particle (SRP) is an essential ribonucleoprotein complex that targets inner membrane proteins (IMPs) to membrane-bound translocons. Since SRP depletion only partially inhibits IMP insertion, however, it is not clear why the particle is critical for cell survival. Insights into this question emerged from experiments in which we analyzed the physiological consequences of reducing the intracellular concentration of SRP below the wild-type level. We found that even moderate SRP deficiencies, which have little effect on cell growth, led to the induction of a heat shock response. Genetic manipulations that suppress the ability of cells to mount a heat shock response were synthetically lethal with moderate SRP deficiencies, indicating that the elevated production of heat shock proteins is required for cell viability. Although it is conceivable that the heat shock response serves to increase the level of molecular chaperones that target IMPs via SRP-independent mechanisms, SRP-deficient cells did not show an increased dependence on either GroEL/ES or DnaK/J. By contrast, the viability of SRP-deficient cells was highly dependent on the presence of heat shock-regulated proteases. Moreover, a model IMP that was mislocalized in the cytoplasm as the result of SRP depletion was rapidly degraded

in control cells but was hyperstabilized in a strain that contains multiple protease mutations. These results provide direct evidence that the heat shock response protects SRP-deficient cells by increasing their capacity to degrade mislocalized IMPs and suggest that SRP has been conserved in prokaryotes in part to prevent protein aggregation in the cytoplasm by maximizing the efficiency of IMP insertion.

S4. Distinct Features of the SRP-Dependent Integration of Bacterial Membrane Proteins

H.G. Koch, K. Beck, C. Neumann-Haefelin, and M. Muller

Institute of Biochemistry and Molecular Biology, University Freiburg, Hermann-Herder-Strasse 7, 79104 Freiburg, Germany.

In addition to SecA and SecB, which are involved in the posttranslational targeting of secretory proteins, bacteria contain homologues of SRP (Ffh and 4.5S RNA) and SRP receptor (FtsY). Recently we have described the first authentic assay system for the bacterial SRP and thereby demonstrated that the integration of polytopic membrane proteins into inside-out membrane vesicles (INV) of *E. coli* exclusively depends on Ffh, FtsY, and 4.5S RNA with no requirement for SecA and SecB. We have now confirmed these results for the three inner membrane proteins of *E. coli*, mannitol permease (MtlA), SecY itself, and a LacY-Bla fusion protein. Moreover, we have obtained evidence that the exclusively SRP-mediated integration of proteins into INV leads to functionally active enzymes. Different from inner membrane proteins, translocation of a secretory protein (pOmpA) into INV is completely independent of SRP requiring only SecA, SecB, and the proton gradient. SRP-dependent integration and SecAB-driven translocation both require an active SecYE translocon whereas SecG is needed only for SecA-mediated translocation. The decision for the SecAB- or the SRP-dependent targeting route is made early during translation at the ribosome. There, nascent chains of inner membrane proteins are specifically recognized by Ffh via a signal anchor sequence. In contrast, interaction of SRP with the signal sequence of a nascent secretory protein is prevented by trigger factor, a ribosome-associated chaperone. Trigger factor also interferes with the binding of SecA to a secretory protein prior to its release from the ribosome. Consistent with a discriminatory action of Ffh and trigger factor, a fusion protein that contains both a signal anchor sequence of an inner membrane protein and the translocated domain of a secretory protein is recognized by both Ffh and trigger factor. Furthermore when still attached to the ribosome, nascent chains of inner membrane proteins are targeted to and maintained in close vicinity to SecY, membrane lipids, and YidC the homologue of Oxal found in inner mitochondrial and thylakoidal membranes.

S5. *Yidc*, the *E. coli* Homologue of Mitochondrial *Oxa1p* is a Component of the Sec Translocase

P. A. Scotti¹, M. L. Urbanus¹, J. Brunner², J. W. de Gier³, G. von Heijne³, C. van der Does⁴, A. J. M. Driessens⁴, B. Oudega¹, J. Luijink¹

¹Department of Microbiology, Institute of Molecular Biological Sciences, Biocentrum Amsterdam, Amsterdam, NETHERLANDS; ²Laboratorium fur Biochemie, Eidgenossische Technische Hochschule Zürich, Zürich, SWITZERLAND; ³Department of Biochemistry, Stockholm University, Stockholm, SWEDEN; ⁴Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, Netherlands

Biochemical and genetic evidence indicates that a subset of *E. coli* proteins are secreted across or integrated into the inner membrane via a SRP-dependent process. The particularly hydrophobic character of the targeting signals of these proteins triggers the specific recognition by the *E. coli* SRP complex. From our previous *in vitro* cross-linking studies we concluded that in the presence of the SRP-receptor *FtsY* and of GTP, the nascent chains are transferred from the SRP to a common translocation site at the inner membrane that contains at least *SecA* and *SecY*. Little is known about the exact topology and consecutive interactions of the nascent chains during their membrane integration. In the present study we further investigated the nature and the precise order of interactions involved in the integration of the model inner membrane protein *FtsQ*. For this purpose, single lysine were introduced at various positions in nascent *FtsQ* 108 mer to serve as site-specific cross-linking using chemical bifunctional cross-linking reagents. A site specific photocross-linking strategy was also used to look specifically at the interaction between the transmembrane domain and lipids. Lysine residues located in between the C-terminal part of the transmembrane domain and the ribosome tunnel exit were strongly cross-linked to both *SecA* and *SecY* suggesting that this segment inserts at *SecA/SecY* interface. As described in eukaryotic systems the transmembrane domain moves out of the translocon during translation as judged by the weak cross-linking efficiency to *SecA/SecY* when lysine residues are introduced within this domain. We demonstrated that the transmembrane domain of *FtsQ* 108 mer is in fact located at an interface between the lipids and surprisingly, a newly characterised protein, *YidC*, which is the *E. coli* homologue of the yeast mitochondrial *Oxa1p*. Furthermore, *YidC* was co-purified with the SecYEG translocon. Taken together these data led us to conclude that *YidC* is part of the *E. coli* translocase possibly acting as a specific receptor for transmembrane domains.

S6. The Mammalian Homolog of the Yeast Sec Complex Subunit Sec63p: Part of a Multifunctional Transport Complex?

J. Tyedmers¹, C. Bies¹, J. Dudek¹, M. Skowronek², I. Haas², W. Nastainczyk¹, J. Volkmer¹, R. Zimmermann¹, M. Jung¹

¹Universität des Saarlandes, Medizinische Biochemie und Molekularbiologie, Homburg, Germany; ²Universität Heidelberg, Biochemie-Zentrum, Heidelberg, Germany

Posttranslational protein transport into the yeast endoplasmic reticulum is mediated by the so called Sec-complex in the ER-membrane, containing a putative signal peptide receptor subcomplex (comprising Sec62p, Sec72p, Sec71p), a Hsp40 type membrane protein, called Sec63p, a protein conducting channel (the Sec61p- complex) plus luminal Hsp70s. Recently, human homologs of the yeast proteins Sec62p and Sec63p were discovered. We determined the concentrations of these two membrane proteins in dog pancreas microsomes and observed that these mammalian homologs are abundant proteins, present in almost equimolar concentrations as compared to the protein conducting channel subunit Sec61alphap. We suggest that Sec62p and Sec63p are parts of a multifunctional transport complex, prepared to function in posttranslational and cotranslational protein transport into as well as protein transport out of the mammalian endoplasmic reticulum.

Immunoprecipitation and purification experiments using microsomal extracts show, that both homologs form a complex with the Sec61p-complex. Furthermore the J-domain of Sec63p interact with the Hsp 70 homolog BiP. We found that the ATPase activity of BiP can be stimulated in a concentration dependent manner by GST-Sec63-J (recombinant J-Domain of Sec63p) and purified recombinant BiP binds to immobilized GST-Sec63-J in the presence of ATP with KD of 5x10-6M. For the luminal Hsp70s BiP and GRP170 it had been shown that they can stimulate cotranslational transport and for BiP to seal non-translocating protein conducting channels. Thus the interaction of Sec63p with the luminal Hsps could raise transport efficiency by directing the transfer of the nascent chain to the lumen or both proteins might be involved in the gating of the translocons. The observations that protein export into the cytosol, delivering proteins to the proteasome for degradation, depends on Sec61alphap and BiP in mammals and on Sec63p in yeast further support our suggestion of Sec63p being a part of a multifunctional transport complex.

S7. Connections Between Nucleoporins, Inositol Signaling, and mRNA Export

Eric Ives, Lisa Strawn, Dianne Barry, Robert Murphy, Audrey Odem, John York, and Susan Wente

Department of Cell Biology and Physiology, Box 8228, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, Missouri 63110 USA

Nuclear pore complexes (NPCs) are large proteinaceous structures that traverse the nuclear envelope and provide the only known portals for transport between the nucleus and cytoplasm. Isolated yeast *Saccharomyces cerevisiae* NPCs are comprised of ~30 different polypeptides, and we have focused our efforts on the study of a family of nucleoporins that share a domain of repetitive tetrapeptide "GLFG" repeats. One aspect of our recent studies have focused on analyzing the network of nucleoporin interactions that result in the assembly of Nup100 and Nup116 at the NPC. By genetic, molecular and biochemical means, we have also found that the GLFG

nucleoporins Nup100 and Nup116 associate with both essential import and export factors during the trafficking mechanism (including karyopherins, Gle1, and Gle2). We have recently identified a novel phospholipase C-dependent inositol polyphosphate kinase pathway that is required for efficient mRNA export, and are determining the role for the second messenger inositol hexakisphosphate in regulating transport.

S8. The Ins and Outs of Transmembrane Protein Folding. Membrane Protein Topogenesis from a Lipid Perspective

M. Bogdanov, W. Dowhan

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Membrane protein topogenesis problem to understand and predict how a given protein sequence will fold itself in a given phospholipid environment. Despite intensive attempts to understand the features of the amino acid sequences controlling the insertion and orientation of membrane protein, the role of the phospholipids as putative topological determinants has been largely ignored. Using combination of site-directed single cysteine (Cys) labeling and site-specific protease (Factor Xa) protection assays, the topology of the largest cytoplasmic loop (C6) of lactose permease (LacY) was explored in phosphatidylethanolamine (PE)-containing and PE-deficient *Escherichia coli* cells and either right side out (RSO) or inside out (ISO) membrane vesicles made from these cells. Two engineered Cys residues or a Factor Xa cleavage site were shown to be fully accessible to a membrane-impermeable biotinylated thiol reagent (MPB) or Factor Xa protease, respectively, when added at the cytoplasmic site of ISO vesicles of PE-containing cells. Simultaneously these tags were not accessible to these agents from the cytoplasmic site of the PE-deficient ISO vesicles, but were fully accessible from periplasmic side of intact cells or RSO vesicles. Moreover, the above agents were able to react with LacY of PE-deficient ISO-vesicles only after permeabilization of the vesicles either with toluene or Triton X100, respectively. However, the C-terminus of LacY remains in the correct orientation regardless the presence of PE. Our data indicate that the absence of PE causes aberrant "flipping" of the cytoplasmic loop C6 into the periplasm and that the remainder of the molecule downstream of this loop exhibits a normal topology. Experiments are underway to elucidate the detailed mechanism of generating an alternative topology of LacY in the absence of PE. Phospholipids appear to be as one of the factors determining the topology of this transmembrane protein.

S9. Component Organization of the Machinery for the Thylakoidal Delta pH/Tat Pathway

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The Delta pH pathway is one of several distinct systems for protein translocation into the thylakoids of plant chloroplasts. This system exhibits several novel characteristics that include a consensus twin-arginine motif in the signal peptide of its substrates, the lack of soluble factor and NTP requirements, and the sole reliance on a transmembrane pH gradient for transport. In addition, the Delta pH pathway and the related

bacterial Tat pathway can transport folded polypeptides. Two components of the Delta pH pathway translocon, Hcf106 and Tha4, have been genetically identified. Three components of the *E. coli* Tat pathway have been identified. TatB is the Hcf106 ortholog, TatA/E is the Tha4 ortholog, and TatC is a multispanning membrane protein. Recently we provided biochemical evidence for the role of Hcf106 and Tha4 in thylakoid protein transport. Here we report that a chloroplast TatC ortholog is required for the Delta pH pathway and describe the organization of components in the membrane. Arabidopsis genomic DNA homologous to TatC was used to isolate a pea chloroplast TatC cDNA. Antibody to the expressed cpTatC amino terminus, when prebound to thylakoids, specifically inhibited protein transport on the Delta pH pathway, but not the Sec or SRP pathways. Blue native gels were used to analyze organization of the components in the membrane. Anti-cpTatC reacted with a 700 kDa complex that was recognized with anti-Hcf106, but not anti-Tha4. Hcf106 also migrated as a 130 kDa species. Tha4 migrated as a 100 kDa species that did not contain Hcf106 or cpTatC. These results were supported by immunoprecipitation studies. Anti-cpTatC co-immunoprecipitated a fraction of Hcf106 and anti-Hcf106 co-immunoprecipitated all of the cpTatC. Tha4 was immunoprecipitated with anti-Tha4, but not with anti-Hcf106 or anti-cpTatC. None of these proteins was co-immunoprecipitated with anti-cpSecY or anti-cpOxa1p, which serve as translocon components of two other thylakoid translocation pathways. These results provide evidence that Hcf106, Tha4, and cpTatC all directly participate in protein transport on the Delta pH pathway. In addition, they suggest a model in which the cpTatC-Hcf106 complex assembles with Tha4 upon establishment of transport conditions to form an active translocon. The exact composition of the translocon may be determined by the size of the protein being translocated. This work was supported by an NIH grant to K.C.

S10. Bacterial Preprotein Translocase: A Dynamic Channel Protein Complex

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Protein translocation across the bacterial cytoplasmic membrane has been extensively studied in *Escherichia coli*. The identification of the components involved and subsequent reconstitution of translocation reaction with purified proteins have defined the minimal constituents that allowed extensive biochemical characterization of the so-called translocase. This functional enzyme complex consist of the SecYEG integral membrane protein complex and a peripherally bound ATPase, SecA. Under translocation conditions, four SecYEG heterotrimers assemble into a large protein complex, forming a putative protein-conducting channel. This tetrameric arrangement

of SecYEG complexes and the highly dynamic SecA dimer together form a proton-motive force and ATP-driven molecular machine that drives the stepwise translocation of targeted polypeptides across the cytoplasmic membrane. Recent findings concerning the translocase structure and mechanism of protein translocation will be discussed.

S11. Projection Structure and Oligomeric Properties of a Bacterial Core Protein Translocase

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The major route for protein export or membrane integration in bacteria occurs via the *sec* dependent transport apparatus. The core inner membrane complex of three proteins *secY*, *secE* and *secG* forms the protein conducting channel, while a soluble enzyme known as *secA* engages substrate and drives translocation across the membrane in an ATP dependent fashion. The *secYEG* complex from *Escherichia coli* has been over-expressed and purified to homogeneity. In detergent solution it exists in association equilibrium of approximately equal proportions of monomeric heterotrimers and tetrameric forms. This observation may reflect a dynamic association from monomer to tetramer, regulated in the inner membrane during protein translocation. Monomers have been crystallized in two dimensions and images recorded by cryo-electron microscopy have been used to calculate a 9 Å projection structure. These crystals contain monomeric *secYEG* related by a *p12_{1,6}* symmetry. The structure reveals features which are consistent with individual -helices with orientations perpendicular to the membrane; other larger electron densities are probably bundles of more tilted transmembrane helical segments. This map provides for the first time a view of a protein translocase.

S12. Cell-Penetrating Peptides, a Tool for Cellular Delivery of Hydrophilic Macromolecules

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The cell plasmamembrane is impermeable for most polar, hydrophilic compounds which lack specific membrane receptors or transporter proteins. Proteins, polypeptides and oligonucleotides are examples of compounds with low biomembrane permeability and, furthermore, they degrade rapidly when inside cells. In order to use these molecules as therapeutic agents the cellular delivery has to be improved. During the past five years, a variety of cell-penetrating peptides like transportan, penetratin and Tat-derived peptides have been demonstrated to translocate into cells. Transportan, a chimera consisting of the neuropeptide galanin 1-12 and the wasp venom peptide mastoparan connected via a lysine, is one of the first peptides which was shown to be cell-penetrating. Biotinylated transportan is added directly into the cell medium. The localization of transportan is determined by indirect immunofluorescence using fluorescently labelled streptavidin, after the fixation and permeabilization of the

cells. The internalization of transportan is not restricted to a specific cell-type and probably not mediated through the endocytosis pathway since the internalization cannot be blocked by either phenylarsine oxide treatment or hyperosmolar sucrose. Internalization was also detected at 4°C which is further evidence for an energy independent mechanism of uptake. Cell-penetrating peptides have successfully been used for delivery of hydrophilic macromolecules into cells. Cargomolecules with several times higher molecular weights than the carrier peptide can be internalized. The cell-penetrating peptides offer a novel technique for cellular delivery into either the cytoplasmic or the nuclear compartments of cells - without disrupting the plasmamembrane.

S13. Structural Basis of Presequence Recognition and Transmembrane Movement During Protein Import into Mitochondria.

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Most mitochondrial proteins are synthesized in the cytosol as precursor proteins with a cleavable N-terminal presequence and are imported into mitochondria. Protein import into mitochondria is mediated by protein assemblies TOM and TIM in the outer and the inner mitochondrial membranes, respectively. In order to understand the molecular mechanisms underlying the multiple functions of the TOM and the TIM complexes in mitochondrial protein import, information on the tertiary structure of each of the TOM/TIM components is essential. In the present study, we have determined the NMR structure of a functional domain of the receptor subunit of the TOM complex, rat Tom20, in complex with a presequence peptide. The bound presequence peptide adopts an amphiphilic α -helical conformation, with a hydrophobic surface that interacts with the hydrophobic patch in the Tom20 groove. The present structure, along with the mutational study carried out on both Tom20 and the presequence, provides insight into the recognition mechanism of the mitochondrial targeting signal by a general import mitochondrial receptor, Tom20.

S14. Targeting of Proteins to the Thylakoid Membrane and the Bacterial Plasma Membrane.

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Several pathways operate for the targeting of newly-synthesised proteins into or across the chloroplast thylakoid membrane and the bacterial plasma membrane. Sec- and SRP-dependent pathways have been extensively characterised in both membranes for soluble and membrane-bound proteins, respectively. However, recent years have witnessed the emergence of additional

pathways with notably different mechanisms. The twin-arginine translocation (Tat) pathway has been shown to operate in thylakoids and numerous bacteria, apparently for the translocation of fully folded proteins. Recent findings on the subunit interactions and mechanism of this system will be presented, following biochemical studies in both *Escherichia coli* and plant thylakoids. In addition, data will be presented on the insertion of polytopic membrane proteins in thylakoids. We have characterised novel types of insertion mechanism that do not rely on either signal recognition particle or membrane-bound Sec apparatus, and have examined the sequence requirements and topology of representative examples. These findings indicate that a high proportion of membrane proteins do not require SRP for insertion into thylakoids, and we discuss the possibility that these proteins may insert spontaneously into the thylakoid.

S15. Selectivity in Sorting of Preassembled Cytosolic PTS1-Proteins in the Yeast *Hansenula polymorpha*

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Peroxisomes are organelles found in all eukaryotic organisms studied so far. Unlike other cellular organelles, their function may be highly diverse, dependent on cell-type and external conditions it encounters. In humans, for instance, they are involved in a great variety of anabolic and catabolic pathways, including plasmalogen and cholesterol biosynthesis as well as -oxidation of very-long chain fatty acids. Peroxisome malfunctioning causes severe inherited disorders, such as Zellweger Syndrome. Peroxisomal matrix proteins are post-translationally sorted from the cytosol. Two targeting signals for peroxisomal matrix proteins have been described; PTS1, a C-terminal tripeptide (SKL and variants) and PTS2, an N-terminal nonapeptide (-RL-X₅-H/QL). Both PTS1 and PTS2 proteins can be imported as folded oligomers. In *Hansenula polymorpha* peroxisomes are massively induced upon growth of this yeast in methanol-containing media. Alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT) reside in these peroxisomes and catalyze the initial steps in methanol-metabolism. All three enzymes are PTS1-proteins and are active as homo-oligomers (AO, octamer; DHAS, dimer; CAT, tetramer). In spite of the fact that folded proteins can be imported into peroxisomes, we found that alcohol oxidase, when assembled in the cytosol, was not incorporated into newly-formed peroxisomes after a shift of a temperature sensitive mutant of *H. polymorpha*, *per13-6^{ts}* (here renamed *pe1-6^{ts}*), defective in peroxisome biogenesis (Waterham *et al.*, 1993). To determine whether this is a general feature for peroxisomal matrix proteins in *H. polymorpha*, we expressed a PTS1-tagged Green Fluorescent Protein in *H. polymorpha pe1-6^{ts}* cells under control of the alcohol oxidase promoter. We found that, in contrast to AO, cytosolic GFP-SKL and also endogenous DHAS were sorted to newly formed peroxisomes at permissive conditions. In addition, reappearance of peroxisomes at permissive temperature also occurred when the

cells were grown in the presence of cycloheximide. These data show that 1) assembled alcohol oxidase is not competent for import into peroxisomes, whereas dihydroxyacetone synthase and GFP-SKL are, and 2) after a shift of *pex1-6*^{ts} cells to permissive conditions, peroxisomes can be assembled from pre-existing proteins, even though no peroxisomal remnants are detected at restrictive conditions.

S16. The Regulation Of Ribosome Exchange On The Endoplasmic Reticulum Membrane.

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In current models of protein translocation in the endoplasmic reticulum (ER), ribosome exchange on the ER membrane serves a principal regulatory function. Thus, ribosome binding to the ER membrane elicits assembly of the translocon, the cohort of proteins that mediate the translocation and early covalent processing of nascent secretory and integral membrane proteins. Subsequently, the termination of protein synthesis yields the dissociation of the ribosomal subunits from the membrane and the coincident inactivation of translocon function. The cycle of ribosome exchange is, therefore, an integral element of the molecular mechanism of protein translocation in the ER. To study the regulation of ribosome exchange on the ER membrane, two experimental approaches were utilized. In one approach, morphometric and biochemical analyses of ribosome-membrane interactions during the protein translation cycle were examined. These experiments were performed in tissue culture cells and examined the state of membrane association following inhibitor based inhibition of elongation (cycloheximide), initiation (pactamycin), or induction of premature chain termination (puromycin). Run-off translation (pactamycin) or premature termination (puromycin treatment) was accompanied by the enhanced release of small ribosomal subunits from the ER membrane. Under these conditions, the majority of the large subunits remained in stable association with the ER membrane. Using *in vitro* systems, we observed that following run-off translation and re-isolation of microsomal membranes, ribosomal subunits remained in stable association with the ER membrane. Membrane-bound ribosomal subunits remaining after run-off translation are capable of initiating protein translation, regardless of whether the mRNA encoded a secretory protein or a soluble, cytoplasmic protein. Membrane-bound ribosomes engaged in the synthesis of soluble, cytoplasmic proteins dissociate during the elongation cycle as intact ribosome/nascent chain complexes. This dissociation reaction thus maintains the compartmental segregation of secretory and cytoplasmic protein synthesis. We conclude that coincident with the termination of protein synthesis membrane-bound small ribosomal subunits become available for the initiation stage of

protein synthesis and large ribosomal subunits remain in stable association with the ER membrane. We propose that physiological ribosome release from the ER membrane requires that the membrane bound ribosome engage in the synthesis of a protein lacking a signal sequence or transmembrane domain.

S17. Subunit Interactions of the *E. coli* Protein Translocase

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Using genetic and biochemical approaches we have been addressing the problems of how the membrane-integrated subunits, SecY, SecE and SecG, interact to make up the translocation channel, and of how the SecA motor protein interacts with the SecYEG membrane complex to drive protein movement. SecY, having 10 transmembrane (TM), 6 cytosolic (C1-C6) and 5 periplasmic domains, is the central component in that it interacts directly with the other translocase subunits. However, its stable existence in the cell is only possible in association with SecE, as unassembled SecY subunit is eliminated by the FtsH protease.

SecA activation. Our mutation studies indicate that the cytosolic domains C5-C6 of SecY are important for activating the SecA ATPase. Among others, Arg357 is the most important residue in this respect.

Dominant negative SecY. Inactive SecY variants due to a mutation in the above domains are invariably dominant-negative as they still bind to SecE and, hence, sequester it.

Cytosolic SecY-SecE interaction. A region including C4 was then identified as a site important for the interaction with SecE, as second site mutations here suppressed the dominant negative phenotype. Residue Gly240 seems to be of particular importance for the SecY-SecE interaction. A SecY-interacting factor called Syd acts to disrupt the SecY-SecE complex that is destabilized by the *secY24* alteration of residue 240, resulting in the abolishment of the high affinity SecA binding.

Intramembrane SecY-SecE interaction. We isolated an omnipotent suppressor mutation in *secE* (in TM3, identical with *prlG2*) that suppressed a number of translocation-retarding mutations in SecY. It is interpreted that this TM mutation makes the channel more open. Consistent with this notion, this *secE* mutation was found to be synthetically lethal with the *secY24* mutation that weakens the SecY-SecE cytosolic interaction. Thus, multiple interactions, both cytosolic and intramembrane, between SecY and SecE are required to make a functional translocation channel.

SecE-SecE interaction and super assembly of SecYEG. Mutations in the cytosolic loop of SecE abolish the SecY-stabilizing ability of this protein, but the mutant proteins were still found to be weakly dominant-negative. Evidence suggested that this interference was due to sequestration of the wild-type SecE molecules. Thus, SecE functions in multimeric states, being consistent with the notion that multiple SecYEG complexes assemble to form a functional channel. We were able to identify a possible SecE site

involved in this super assembly, as a site of intragenic suppressor mutation.

A SecY-SecG interaction site. We have identified a secY mutation in C3 domain that can be suppressed allele-specifically by secG mutations. Taken together with our results of site-specific crosslinking experiments, C3 region of SecY is suggested to be a SecG-interacting site.

SecY and insertion of SecA. We have identified a secY mutation (in C6) that specifically impaired the ATP- and preprotein-dependent mode of SecA insertion reaction. We have identified a large number of secA mutations, both allele-specific and omnipotent (or "super active") that suppressed this secY defect. Our results support the notion that the SecA insertion is indeed important for translocation and that SecA must interact properly with SecY in order to carry out the insertion-deinsertion cycles.

S18. Site-Specific Phosphorylation Used as a Marker for Membrane Protein Topology and as a Timer to Estimate the Time-Course of SRP-Dependent Protein Targeting to the Endoplasmic Reticulum

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We have developed a novel procedure to determine in vivo the cytosolic localization of membrane protein sequences by introducing a heptapeptide consensus sequence for specific phosphorylation by cAMP-dependent protein kinase. This phosphorylation tag could furthermore be used to assess the dynamics of protein targeting and translocation. The phosphorylation site was positioned at the N-terminus of a type III (Nexo/Ccyt) membrane protein which is only transiently exposed to the cytosol prior to its translocation into the lumen of the endoplasmic reticulum (ER). We found that the degree of phosphorylation increased with increasing length of the spacer between the phosphorylation site and the internal signal-anchor sequence from 20 up to 80 amino acids, reflecting the time the tag was exposed to the cytosol before reaching the ER lumen. Extrapolation of the phosphorylation intensity to a spacer of zero length gives an estimate of the time required for SRP binding and targeting to the ER membrane. The results show that this process is quite rapid compared to the rate of translation, suggesting that — at least for the signal sequence tested — translation arrest is not necessary to allow targeting.

S19. Unique Chaperone Requirements Define the ER Associated Degradation (ERAD) of Soluble and Membrane Proteins

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Post-translational translocation into the yeast ER requires at least two hsp70s, Ssa1p in the cytosol and BiP (Kar2p) in the ER lumen. After import, aberrant polypeptides may be exported to the cytoplasm for degradation by the proteasome, in a process we have termed ER Associated Degradation (ERAD). Both import and export require BiP and the Sec61p translocation complex, suggesting that import and export may be mechanistically related. We

found, however, that the export and degradation of two soluble ERAD substrates is defective in kar2 mutant microsomes that are proficient for polypeptide import. To examine whether Ssa1p might drive polypeptides from the ER for degradation, we measured ERAD in strains either containing a temperature-sensitive mutation in SSA1 or in which the level of Ssa1p could be regulated. Surprisingly, ERAD was proficient both in vivo and in vitro under conditions in which Ssa1p activity was compromised. These results indicate that the mechanisms of import into and export from the ER of soluble polypeptides are distinct. To examine the ERAD of an integral membrane protein, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) was expressed in yeast. In the ssa1 strain, degradation was attenuated at the non-permissive temperature, whereas CFTR degradation was unaffected in the kar2 strain. Our combined data indicate reciprocal chaperone requirements for the proteolysis of integral membrane and soluble ERAD substrates, and reveal the complexity underlying the selection and targeting of polypeptides to the proteasome.

S20. A Microsomal GTPase is Required for Glycopeptide Export from Mammalian Endoplasmic Reticulum

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Bidirectional transport of proteins via the Sec61p translocon across the endoplasmic reticulum membrane is a recognized component of the ER quality control machinery. Following translocation and engagement by the luminal quality control system, misfolded and unassembled proteins are exported from the ER lumen back to the cytosol for degradation by the proteasome. Additionally, other ER contents, including oligosaccharides, oligopeptides and glycopeptides are efficiently exported from mammalian and yeast systems indicating that bidirectional transport across ER membranes is a general eukaryotic phenomenon. Glycopeptide and protein export from ER in *in vitro* systems is both ATP and cytosol-dependent. Using a well established system to study glycopeptide export from eukaryotic ER membranes (Romisch, K. & B.R.S. Ali, 1997, *Proc. Natl. Acad. Sci. USA*, 94: 6730-6734) and conventional liquid chromatography, we isolated a single polypeptide species of 23 kDa from rat liver cytosol that was capable of fully supporting glycopeptide export from rat microsomes in the presence of an ATP-regenerating system. The protein was identified by mass spectrometric sequence analysis as guanylate kinase, a housekeeping enzyme critical in the regulation of cellular GTP levels. We confirmed the ability of GK to substitute for complete cytosol by reconstitution of glycopeptide export from rat liver microsomes using highly purified recombinant GK from *Saccharomyces cerevisiae*. Most significantly, we found that GK, and hence cytosolic components, requirement was fully bypassed by low micromolar concentrations of GDP or GTP. Similarly, export was inhibited by nonhydrolysable analogues of GDP or GTP indicating a requirement for GTP hydrolysis. Membrane integrity was fully maintained under assay conditions as no luminal ER proteins were released. Competence for glycopeptide export was abolished by very

mild trypsin treatment of microsomes indicating the presence of an essential protein on the cytosolic face of the ER membrane. These data demonstrate that glycopeptide export from rat liver microsomes is controlled by a microsomal GTPase and is independent of cytosolic protein factors.

S21. Filamentous Phage f1 are Transported Across Membranes Through Phage-Encoded Aqueous Channels

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Purpose: Proteins can only cross the endoplasmic reticulum, mitochondrial, chloroplast or inner bacterial membranes as an unfolded linear polymer. However, many macromolecules fold, and even oligomerize, before they cross the outer bacterial membrane and they cross without compromising the integrity of the membrane. One such example is filamentous phage f1 which exits its *Escherichia coli* host as a 70A diameter cylinder. The phage-encoded protein pIV, which shares homology with many other proteins involved in toxin export across the outer membranes of *Salmonella*, *Shigella*, *Yersinia*, and *Vibrio cholerae*, is required for this transport. We examine how pIV transports phage without compromising the integrity of the bacterial membrane. Specifically, the goal is to test if pIV forms a transmembrane aqueous channel for the transport of filamentous phage. **Methods and Materials:** The potential membrane transport activity of pIV was examined both *in vitro* (purified and reconstituted into planar lipid bilayers) and *in situ* (by examining transport of large macromolecules through pIV in the living *E. coli*). **Results:** Purified and reconstituted pIV protein forms a large channel in lipid bilayers. The biophysical properties of the channel are affected by single point mutations in the pIV gene (demonstrating that the channel is due to pIV and not a co-purifying contaminant). In the intact bacteria, pIV increases the permeability of the outer membrane to macromolecules. Under normal physiological conditions the pIV channels are closed. However, point mutations that increase the probability of the channel opening in the *in vitro* electrophysiological assays also increase the probability of the channel opening *in situ*. Transport of macromolecules through these channels is blocked during phage export. **Conclusions:** pIV forms a large (>6 nm) channel in the outer membrane through which phage are extruded. This is the first demonstration of such a channel in the type II and type III secretion systems in bacteria and the first demonstration that the channels are used for export.

S22. Co-translational Interactions of Apoprotein B with the Ribosome and Translocon During its Assembly with Lipids in the Endoplasmic Reticulum or its Targeting to Proteasomal Degradation in the Cytosol

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Apoprotein B (apoB) is a major hepatic protein that assembles with lipids in the ER to form the atherosclerosis-causing lipoprotein particles secreted into the plasma. A crucial regulatory step in hepatic lipoprotein assembly and secretion is the proteasomal degradation of newly synthesized apoB, which increases if the rate of lipid synthesis or lipid transfer is low, resulting in a deficiency of "lipid-ligands". Our previous studies in the human hepatic carcinoma cell line HepG2 showed that when proteasomal activity is inhibited when lipid synthesis is low, newly synthesized apoB remains stably associated with the ER membrane. We now show that independent of lipid synthesis, nascent apoB chains that appear full-length are, in fact, incompletely translated polypeptides still engaged by the ribosome and associated with the ER translocon protein sec61-alpha. In the presence of active lipid synthesis and transfer, translation and lipoprotein assembly complete, and the interactions with the ribosome and translocon are lost as the apoB-lipid complexes assembly exit the ER. In the absence of lipid synthesis or transfer, apoB undergoes degradation while remaining associated with the ER membrane, as reflected by interactions with cytosolic hsp70 and proteasomes. Thus, unlike other ER-substrates of the proteasome, such as MHC class I molecules in CMV-infected cells and mutant carboxypeptidase Y in yeast, apoB does not fully retrotranslocate to the cytosol before entering the ubiquitin-proteasome pathway. Another distinguishing feature between apoB and other substrates of the proteasome is the lack of aggresomal formation in the cytosol when the proteasome is inhibited. Although by immunofluorescence apoB accumulates in punctate structures similar in appearance to aggresomes, unlike the latter, these are reversible. In summary, the results suggest a model in which lipoprotein assembly or entry into the ubiquitin-proteasomal pathway occurs while apoB is still associated with the translocon and attached to the ribosome.

POSTER SESSION ABSTRACTS

1. Length recognition at the N-terminal tail of membrane proteins as a mode of initiation of FtsH-mediated proteolysis of membrane proteins

Shinobu Chiba, Yoshinori Akiyama, Hiroyuki Mori, Eiichi Matsuo and Koreaki Ito

FtsH-mediated proteolysis against membrane proteins is processive, and presumably involves dislocation of the substrate into the cytosol where the enzymatic domains of FtsH reside. To study how such a mode of proteolysis is initiated, we manipulated N-terminal cytosolic tails of three membrane proteins. YccA, a natural substrate of FtsH was found to require the N-terminal tail of 20 amino acid residues or longer to be degraded by FtsH *in vivo*. Three unrelated sequences of this segment conferred the FtsH-sensitivity to YccA. An artificially constructed TM9-PhoA protein, derived from SecY, as well as the SecE protein were sensitized to FtsH by addition of extra amino acid sequences to their N-terminal cytosolic tails. Thus, FtsH recognizes a cytosolic region of sufficient length (about 20 amino acids) to initiate the processive proteolysis against membrane proteins. Such a region is typically at the N-terminus and can be diverse in amino acid sequences.

2. *YidC*, the *E. coli* Homologue of Mitochondrial *Oxa1p* is a Component of the *Sec* Translocase

P. A. Scotti¹, M. L. Urbanus¹, J. Brunner², J. W. de Gier³, G. von Heijne³, C. van der Does⁴, A. J. M. Driessens⁴, B. Oudega¹, J. Luirink¹

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Biochemical and genetic evidence indicates that a subset of *E. coli* proteins are secreted across or integrated into the inner membrane via a SRP-dependent process. The particularly hydrophobic character of the targeting signals of these proteins triggers the specific recognition by the *E. coli* SRP complex. From our previous *in vitro* cross-linking studies we concluded that in the presence of the SRP-receptor *FtsY* and of GTP, the nascent chains are transferred from the SRP to a common translocation site at the inner membrane that contains at least *SecA* and *SecY*. Little is known about the exact topology and consecutive interactions of the nascent chains during their membrane integration. In the present study we further investigated the nature and the precise order of interactions involved in the

integration of the model inner membrane protein *FtsQ*. For this purpose, single lysine were introduced at various positions in nascent *FtsQ* 108 mer to serve as site-specific cross-linking using chemical bifunctional cross-linking reagents. A site specific photocross-linking strategy was also used to look specifically at the interaction between the transmembrane domain and lipids. Lysine residues located in between the C-terminal part of the transmembrane domain and the ribosome tunnel exit were strongly cross-linked to both *SecA* and *SecY* suggesting that this segment inserts at *SecA/SecY* interface. As described in eukaryotic systems the transmembrane domain moves out of the translocon during translation as judged by the weak cross-linking efficiency to *SecA/SecY* when lysine residues are introduced within this domain. We demonstrated that the transmembrane domain of *FtsQ* 108 mer is in fact located at an interface between the lipids and surprisingly, a newly characterised protein, *YidC*, which is the *E. coli* homologue of the yeast mitochondrial *Oxa1p*. Furthermore, *YidC* was co-purified with the *SecYEG* translocon. Taken together these data led us to conclude that *YidC* is part of the *E. coli* translocase possibly acting as a specific receptor for transmembrane domains.

3. Cell-Penetrating Peptides, a Tool for Cellular Delivery of Hydrophilic Macromolecules

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The cell plasmamembrane is impermeable for most polar, hydrophilic compounds which lack specific membrane receptors or transporter proteins. Proteins, polypeptides and oligonucleotides are examples of compounds with low biomembrane permeability and, furthermore, they degrade rapidly when inside cells. In order to use these molecules as therapeutic agents the cellular delivery has to be improved. During the past five years, a variety of cell-penetrating peptides like transportan, penetratin and Tat-derived peptides have been demonstrated to translocate into cells. Transportan, a chimera consisting of the neuropeptide galanin 1-12 and the wasp venom peptide mastoparan connected via a lysine, is one of the first peptides which was shown to be cell-penetrating. Biotinylated transportan is added directly into the cell medium. The localization of transportan is determined by indirect immunofluorescence using fluorescently labelled streptavidin, after the fixation and permeabilization of the cells. The internalization of transportan is not restricted to a specific cell-type and probably not mediated through the endocytosis pathway since the internalization cannot be blocked by either phenylarsine oxide treatment or hyperosmolar sucrose. Internalization was also detected at 4°C which is further evidence for an energy independent mechanism of uptake. Cell-penetrating peptides have

successfully been used for delivery of hydrophilic macromolecules into cells. Cargomolecules with several times higher molecular weights than the carrier peptide can be internalized. The cell-penetrating peptides offer a novel technique for cellular delivery into either the cytoplasmic or the nuclear compartments of cells - without disrupting the plasmamembrane.

4. Structural Requirements for the Correct Membrane Integration of a Seven-helix Receptor

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In membrane proteins with multiple membrane spanning segments, it is not yet firmly established whether the commitment to the correct orientation occurs soon after the synthesis of the first transmembrane segment, or whether it occurs at a later point in biogenesis. Here, we present our deductions on the mechanism of membrane protein biogenesis based on an analysis of the steady-state membrane orientation of a series of C-terminal helix deletion mutants of the seven-helix membrane protein rhodopsin expressed in COS-1 cells. Full-length bovine rhodopsin is glycosylated at two asparagine residues at the N terminus and displays an Nexo-Ccyto membrane orientation. Trypsin digestion studies with microsomes isolated from cells expressing opsin mutants with three or fewer transmembrane segments show that the N terminus is preferentially proteolyzed, while the C-terminal end is partially or completely protected. Surprisingly, this pattern of reactivity is observed with both unglycosylated and glycosylated forms of the polypeptides. We interpret these results as evidence for a steady-state topology in the shorter constructs that is opposite to that seen in full-length opsin. We conclude that the presence of the C-terminal set of transmembrane segments plays a crucial role in determining the final orientation of opsin, and that N-terminal glycosylation, which we assume occurs solely in the lumen of the endoplasmic reticulum, does not guarantee that the shorter polypeptides will continue to maintain an Nexo-Ccyto orientation in the membrane of the endoplasmic reticulum. Our results suggest that newly synthesized membrane proteins may have considerable motional flexibility inside the translocon and that the commitment to the correct orientation in a multispanning membrane protein only occurs after the synthesis of several transmembrane segments.

5. *Yersinia* YopE is Targeted for Type III Secretion by an Amphipathic Sequence at its Amino-terminus

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Pathogenic *Yersinia* species inject antiphagocytic virulence proteins, known as Yops, into the cytosol of eukaryotic cells. The injection of Yops is mediated by a type III secretion system. Yop proteins do not exhibit significant sequence similarities. Previous studies have suggested that Yops are targeted to the secretion system by an RNA-dependent mechanism. It has been proposed that

approximately the first 15 codons of *yop* mRNAs, together with upstream untranslated RNA sequences, form stem-loop structures that are recognized by the secretion system. In this model, the expression of Yops is repressed due to the sequestration of Shine-Dalgarno sequences within the stems of such structures. Repression is relieved upon interaction of these RNA structures with the type III secretion system, thereby coupling secretion and translation. The existence of *yop* mRNA targeting signals is controversial, however, as a prior mutational analysis has shown that the first seven amino acid residues of YopE are important for secretion. In order to better characterize the nature of *Yersinia* type III secretion signals, we systematically mutagenized the first 11 codons of *yopE*. Frameshift mutations were introduced immediately after the initiation codon and compensatory mutations were introduced after codon 11 to restore the reading frame. YopE mutants were expressed from a plasmid in a *yopE* null strain such that the *yopE* mRNA transcript contained heterologous untranslated RNA sequences. Expression of YopE was under the control of an arabinose-inducible promoter. Upon induction, the secretion of YopE, both in liquid cultures and in a eukaryotic cell infection assay, was measured using an anti-YopE antisera. We report that frameshift mutations, which completely alter the amino acid sequence of residues two through eleven but leave the RNA sequence essentially intact, significantly reduce the secretion of YopE. In contrast, mutations that alter the *yopE* mRNA sequence and its predicted stem-loop structure, while leaving the amino acid sequence of YopE unchanged, do not impair the secretion of YopE. Replacement of the native YopE amino-terminus with heterologous amino acid sequences of differing physical properties revealed that an amphipathic sequence of six to eight residues is sufficient to serve as a targeting signal. We conclude that the amino-terminus of YopE, and not the 5' end of *yopE* mRNA, is recognized by the *Yersinia* type III secretion system.

6. Determinants of SecA-Signal Peptide Interactions

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SecA is considered a critical component of the bacterial translocon which provides the energy, through ATP hydrolysis, for preprotein membrane translocation. It interacts specifically with signal peptides, in the absence of the mature region of the protein (Miller et al., 1998, J. Biol. Chem. 273, 11409-11412). SecB is a cytosolic chaperone which is believed to facilitate the membrane targeting step by delivering the preprotein to SecA. Elucidating the determinants of the SecA-signal peptide interaction will provide a better understanding of the preprotein interaction with the transport machinery and the role that SecA plays in the translocation process. To study the SecA-signal peptide interaction *in vitro*, a series of synthetic model signal peptides, with known *in vivo* function, were made. These include the wild type alkaline phosphatase signal peptide and three pairs of model signal peptides which vary in the hydrophobicity of the core region. Within each pair, the amino terminal charge is also different. The effects of these model signal peptides on SecA/lipid ATPase activity, SecA V8 protease sensitivity, and

the extent of wild type signal peptide crosslinking to SecA were studied. The SecA/lipid ATPase activity assay demonstrated that those signal peptides with a highly hydrophobic core region markedly stimulated activity while those with a minimally hydrophobic core gave little or no activity, regardless of the amino terminal charge. The signal peptides with intermediate hydrophobicity produced an intermediate level of activity but the activity was higher for the one with more positive charges at the N-terminus. The crosslinking displacement assay and the SecA V8 digestion assay substantiated this pattern of signal peptide features which promote interaction with SecA in solution and in liposomes. In addition, SecB caused a significant increase in the ATPase activity produced by all the peptides. The results indicate that the signal peptide-SecA interaction primarily involves hydrophobic-hydrophobic contacts. However, electrostatic interactions are also involved although the contribution is secondary and is only apparent when the hydrophobic interaction is marginally satisfied. The parallel between the properties required for the SecA-signal peptide interaction *in vitro* and the ability to support preprotein transport *in vivo* suggests that such an interaction may be the rate-limiting step for preprotein transport overall.

7. The Soluble Mitochondrial TOM Complex Can Bind and Translocate Precursor Proteins

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A multisubunit complex in the mitochondrial outer membrane (TOM complex) is responsible for targeting and membrane translocation of nuclear-encoded preproteins. We have isolated TOM holo complex containing the preprotein receptor components Tom70 and Tom20, and TOM core complex lacking these receptors. The binding of mitochondrial precursor proteins to both types of soluble TOM complex was analyzed. Recombinant preproteins were found to bind efficiently to the soluble complex in the absence of chaperones and lipids in a bilayer structure. A surprisingly high number of precursor molecules (7-9) were found to be bound to one TOM core complex. The binding was salt dependent and could take place even after proteolytic removal of soluble domains of TOM components. Using fluorescently labeled precursor and fluorescent correlation spectroscopy (FCS), a binding constant in the nM range was determined. Following the initial binding, the presequence part of the preproteins is transferred into the translocation pore in a step that required unfolding of the mature part of the preprotein. The translocation step can be mediated also by a protease treated TOM holo complex which contains almost exclusively Tom40, but not by the TOM core complex. These results suggest that the TOM complex represents the minimal machinery for the recognition and partial translocation of mitochondrial precursor proteins.

8. The SH3 Domain of the Peroxisomal Membrane Protein Pex13p Interacts with the PTS1 Receptor in an Unconventional, Non-PXXP Related Manner

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Proteins that reside in the peroxisomal matrix are synthesized on free polyribosomes in the cytosol and posttranslationally imported into the peroxisome. These matrix proteins contain peroxisomal targeting sequences (PTSs) that are specifically recognized by their matching soluble receptor Pex5p (for PTS1 proteins) or Pex7p (for PTS2 proteins). Receptors with bound PTS proteins converge on a common translocation machinery. Two proteins of this complex, Pex13p and Pex14p, physically interact with Pex5p and Pex7p, implying a role in docking of the receptors. Pex13p not only binds Pex5p but also interacts with Pex14p. Both interactions are mediated by the SH3 domain of Pex13p. Pex14p contains the classical SH3 binding motif PXXP, whereas this sequence is absent in Pex5p. Here we demonstrate that Pex5p represents a novel, non-PXXP ligand. Using alanine scanning, two-hybrid and *in vitro* analysis we show that an alpha-helical element in Pex5p is necessary and sufficient for SH3 interaction. Pex5p containing mutations in this helical element could only partially restore PTS1 protein import *in vivo*. Suppressor analysis using Pex5p mutants located in this alpha-helical element allowed the identification of a unique site of interaction for Pex5p on the Pex13p-SH3 domain, that is distinct from the classical P-X-X-P binding pocket. On the basis of a structural model of the Pex13p-SH3 domain we show that this interaction probably takes place between the RT and distal loops. Thus, the Pex13p-SH3-Pex5p interaction establishes a novel mode of SH3 interaction that is required for efficient PTS1 protein import into peroxisomes.

9. The Ins and Outs of Transmembrane Protein Folding. Membrane Protein Topogenesis from a Lipid Perspective

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Membrane protein topogenesis problem to understand and predict how a given protein sequence will fold itself in a given phospholipid environment. Despite intensive attempts to understand the features of the amino acid sequences controlling the insertion and orientation of membrane protein, the role of the phospholipids as putative topological determinants has been largely ignored. Using combination of site-directed single cysteine (Cys) labeling and site-specific protease (Factor Xa) protection assays, the topology of the largest cytoplasmic loop (C6) of lactose permease (LacY) was explored in phosphatidylethanolamine (PE)-containing and PE-deficient *Escherichia coli* cells and either right side out (RSO) or inside out (ISO) membrane vesicles made from these cells. Two engineered Cys residues or a Factor Xa cleavage site were shown to be fully accessible to a membrane-impermeable biotinylated thiol reagent (MPB) or Factor Xa protease, respectively, when added at the cytoplasmic site of ISO vesicles of PE-containing cells. Simultaneously these tags were not accessible to these agents from the cytoplasmic site of the PE-deficient ISO vesicles, but were fully accessible

from periplasmic side of intact cells or RSO vesicles. Moreover, the above agents were able to react with LacY of PE-deficient ISO-vesicles only after permeabilization of the vesicles either with toluene or Triton X100, respectively. However, the C-terminus of LacY remains in the correct orientation regardless the presence of PE. Our data indicate that the absence of PE causes aberrant "flipping" of the cytoplasmic loop C6 into the periplasm and that the remainder of the molecule downstream of this loop exhibits a normal topology. Experiments are underway to elucidate the detailed mechanism of generating an alternative topology of LacY in the absence of PE. Phospholipids appear to be as one of the factors determining the topology of this transmembrane protein.

10. Sls1p Promotes Recruitment of Kar2p by Sec63p in a Conformation Dependent Manner in the Yeast Endoplasmic Reticulum

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We report significant data on the function of Sls1p, a new class of Hsp70 cofactor, that is shown to act along with Kar2p and Sec63p in the translocation process. We previously characterized the SLS1 gene product in the yeast *Yarrowia lipolytica*, and showed that it interacts with Kar2p to promote protein translocation across the ER membrane (Boisrame et al., 1998). Sls1p was shown to interact with the ATPase domain of Kar2p (Kabani et al., 2000) suggesting a regulatory role on the molecular chaperone function. A two-hybrid screen allowed us to identify a Kar2p mutation (G234R) that restored interaction with a mutant form of Sls1p. This mutation was similar to previously identified mutations in *Saccharomyces cerevisiae* (McClellan et al., 1998) or hamster BiP (Wei et al., 1995) that affect ATP binding. A working hypothesis was that Sls1p's interaction with Kar2p is nucleotide and/or conformation dependent. To test this hypothesis and gain better insight in Sls1p's function, we started to work with the Sls1p homologue in *S. cerevisiae*. Synthetic lethality was observed when ScSLS1 was disrupted in several kar2 or sec63-1 mutants, providing *in vivo* evidence for a role of Sls1p in protein translocation, and strongly suggesting that its functions, as for Kar2p, are extended to folding, quality control and protein degradation. Using dominant lethal Kar2p mutations (McClellan et al., 1998), we show by two-hybrid and *in vitro* binding assays that ScSls1p preferentially interacts with the ADP-bound conformation of Kar2p. Moreover, we show that ScSls1p stimulates in a dose dependent manner the binding of Kar2p to the J-domain of Sec63 fused to GST, and remains bound to the GST-63J.Kar2p complex. These results suggest either that Sls1p may promote the Sec63p mediated recruitment of Kar2p to the translocation, or that it stabilizes the interaction of Kar2p with its substrates. These hypothesis are now tested and will be presented.

11. Functional Analysis of Ankyrin and Chromatin Domains of Protein cpSRP43 of the Chloroplast Signal Recognition Particle

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Chloroplast signal recognition particle (cpSRP) is a 200 kDa multimer that functions in targeting light harvesting chlorophyll proteins (LHCPS) to the thylakoid membrane via a novel post-translational mechanism. The 200 kDa particle consists of a 54 kDa GTPase (cpSRP54) and a dimer of cpSRP43, a 43 kDa protein of no known homology. CpSRP43 appears to be an adapter protein capable of engaging in several simultaneous protein-protein interactions, coordinating the soluble post-translational protein targeting complex (transit complex) in the stroma. This 43 kDa subunit has recently been found to participate in at least three concurrent protein-protein interactions: binding to 1) cpSRP54, 2) a specific 18 amino acid region of LHCPS, and 3) itself as a homo-dimer. SMART analysis of the primary sequence revealed that the protein structure is likely composed of mainly ankyrin (Ank) and chromatin-binding (Chromo) functional domains. These domains are known to mediate protein-protein interactions and often appear in repeated units as in cpSRP43, which contains three of each. To understand the role of these domains in transit complex formation, we deleted the domains and subsequently analyzed them by chromatographic, native PAGE, and GST pull-down assays. Three separate deletions (DAnk2, DAnk3, and DChromo3) were constructed by removing ~50aa from each domain and expressing as a GST fusion protein. These mutated proteins were incubated with radiolabeled cpSRP54 and LHCPS, bound to glutathione sepharose, washed, and eluted. The eluate was then analyzed by SDS-PAGE and phosphorimaging to assess binding efficiency of the deletion construct to either LHCPS or cpSRP54. Each construct was also cleaved from its GST fusion and assayed for loss of dimerization by size exclusion FPLC. Transit complex assays were performed by *in vitro* complex formation and analysis by native PAGE. Our data indicate that none of the individual deleted domains of cpSRP43 are required for protein-protein interactions with either LHCPS or cpSRP54 alone, however DChromo3 had a profound effect on formation of transit complex. These results suggest a more elegant and coordinated binding event involving cpSRP43, LHCPS, and cpSRP54 than previously thought.

12. The Peroxisome Biogenesis Factors Pex4p, Pex22p, Pex1p, and Pex6p Act in the Terminal Steps of Peroxisomal Matrix Protein Import

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Peroxisomes are independent organelles found in virtually all eukaryotic cells. Genetic studies have identified more than twenty *pex* genes that are required for peroxisome biogenesis. The role of most *pex* gene products, peroxins, remains to be determined but a variety of studies have established that Pex5p binds the type-1 peroxisomal targeting signal and is the physical receptor for most newly synthesized peroxisomal matrix proteins. In the yeast *Pichia pastoris*, the steady-state

abundance of Pex5p is unaffected by most *pex* mutants but is severely reduced in *pex4* and *pex22* mutants and moderately reduced in *pex1* and *pex6* mutants. We used these subphenotypes to determine the epistatic relationships among several groups of *pex* mutants. Our results demonstrate that Pex4p acts after the peroxisome membrane synthesis factor Pex3p, the Pex5p docking factors Pex13p and Pex14p, the matrix protein import factors Pex8p, Pex10p and Pex12p, and two other peroxins, Pex2p and Pex17p. Additionally, we find that Pex10p acts before Pex22p and the interacting AAA ATPases Pex1p and Pex6p but Pex1p and Pex6p act upstream of Pex4p and Pex22p. These results suggest that Pex1p, Pex4p, Pex6p, and Pex22p act late in peroxisomal matrix protein import after matrix protein translocation. This hypothesis is supported by the phenotypes of the corresponding mutant strains. As has been shown previously for the *P. pastoris* *pex1*, *pex6*, and *pex22* deficient cells, we show here that *pex4δ* cells contain numerous PMP-containing peroxisomes that import residual amounts of peroxisomal matrix proteins.

13. Conformational Regulation of SecA Nucleotide Binding and its Alteration by azi and prlD Mutations

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SecA's ATPase activity is critical for the translocation of preprotein through the *Escherichia coli* inner membrane. To understand this activity we characterized the nucleotide binding of SecA dimer in solution. Purified protein was incubated with nucleotides and the resulting complexes were analyzed using the nitrocellulose filter binding method, gelfiltration and equilibrium dialysis. The dissociation constant for ADP measured by these methods at 4°C amounted between 110 and 374 nM. Each SecA homodimer bound to one molecule of ADP at ADP concentrations less than 100 mM. This nucleotide-binding affinity was conformationally regulated by temperature: at low temperature SecA affinity for ADP was high with a slow exchange rate, while at high temperature the converse was true. In order to characterize the ATP binding behavior of SecA dimer we performed competition assays with ADP. We observed that SecA preferentially binds to ADP over ATP. SecA-D209N, a mutant defective in ATPase activity bound to ADP with low affinity. ATP binding and hydrolysis could not be observed but upon addition of ADP ATPase activity was induced. Azi- and PrlD-SecA proteins that confer azide resistant and signal sequence suppressor phenotypes were found to have reduced affinity for ADP and accelerated exchange rates compared to wildtype SecA. The level of Azi- and PrlD-SecA protein was also elevated in inverted membrane vesicles. These results provide the first direct evidence for conformational regulation of the SecA-dependent nucleotide cycle, its alteration in azi and prlD mutants, and its relevance to *in vivo* protein export.

14. Identification of Amino Acid Residues of SecY that is Important for Activation of SecA

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The SecA protein (preprotein-driving ATPase) and the SecYEG complex (putative channel forming unit) have essential roles in protein translocation across the cytoplasmic membrane of *Escherichia coli*. SecY, the central membrane-integrated subunit, has 10 transmembrane, 5 periplasmic and 6 cytoplasmic regions. Results of our genetic and biochemical studies suggest that the fifth cytoplasmic domain (C5) of SecY is important for the functioning of the SecA ATPase. A *secY* mutation, *secY39* (Arg357His) in this domain, severely impairs protein export *in vivo* and SecA activation *in vitro*. In this study, we first isolated 20 *secA* mutations as suppressors against *secY39*. All the mutants, with a single amino acid substitution in or around the secondary ATP-binding domain, proved to be omnipotent in suppressing different cold-sensitive *sec* mutations, and these SecA variants are called "super-active" SecA. We purified 9 of them and examined their *in vitro* activities. They were all found to possess unusually high ATPase activities and to compensate for the *in vitro* translocation defects of membrane vesicles from several *secY* mutants including *secY39*. We then mutagenized each residue of a segment of Ser349 to Tyr365 in C5, using oligonucleotide linkers synthesized with mixed bases at the target position. Altogether, more than 200 amino acid substitutions were assigned either functional or nonfunctional/dominant-negative. It was shown collectively that the different residues in this region have different degrees of compatibility with replacements by other amino acids. Since substitutions at Arg357 most easily inactivated the SecY function, this residue seemed of particular importance. The dominant negative effects were found to be suppressible by the "super-active" mutations in SecA. From these results we propose that the ATPase activity of SecA is tightly regulated by the proper SecY-SecA interaction, that the secondary ATP-binding region of SecA is involved in the down regulation of ATPase activity, and that Arg357 participates in the activation of SecA.

15. Analysis of Type III Secretion and Delivery System of Pathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC) inserts a bacterial protein, Tir, using a type III secretion system, into the mammalian host cell where it functions as the receptor for the bacterial outer membrane protein intimin to trigger intimate attachment. Detergents are frequently used to fractionate infected host cells to investigate bacterial protein delivery into mammalian cells. In this study, we found that the Triton X-100 soluble membrane fraction from EPEC-infected HeLa cells was contaminated with bacterial proteins. We therefore applied a mechanical method of cell lysis and ultracentrifugation to fractionate infected HeLa cells to investigate the biology and biochemistry of Tir delivery and translocation. This method demonstrates that the translocation of Tir into the host cell membrane requires its transmembrane

domains, but not tyrosine-phosphorylation or binding to Tir's ligand, intimin. The role of the type III secretion system in Tir delivery into the host cell was further investigated using defined mutants of the type III secretion apparatus. These mutants include deletions in the genes encoding EscN, hypothesized to supply ATP for protein secretion, EscV, which is predicted to be an inner membrane protein, and EscC, predicted to be an outer membrane secretin. Bacterial fractionation revealed that Tir and other secreted proteins accumulate in the cytosol of the type III mutants, whereas these proteins are secreted by parental EPEC. Since type III secretion systems are being found in an increasing number of animal and plant pathogens, these studies will not only elucidate how EPEC delivers its virulence factors into host cells, but will also impact the understanding of type III systems in general.

16. The Sorting Sequence of Cytochrome b₂ Influences the Delta Psi-Dependence of Mitochondrial Protein Import in a Charge-Independent Manner

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The mitochondrial membrane potential delta psi is essential for the translocation of preproteins into or across the mitochondrial inner membrane. Both effects that have been described for the role of delta psi in the import of preproteins are related to the positively charged sequences of these proteins. Since the membrane potential is negative on the inner side of the inner membrane, it generates an electrophoretic force on the positive charges. In addition delta psi influences the dimerisation of the translocase subunit Tim23 which is a prerequisite for binding of matrix targeting sequences. Using cytochrome b₂ fusion-proteins, we made the unexpected observation that the sorting sequence, which is located behind the matrix targeting sequence, strongly influenced the delta psi-dependence of import. A deletion in this segment containing several positive charges led to an increased delta psi-dependence of import. The difference in requirement for delta psi was independent of the submitochondrial destination and not related to a differential requirement for mitochondrial Hsp70 or Tim23. Following this observation a series of different preproteins with exchanges of charged amino acids in the sorting sequence against neutral residues was constructed. The import of these constructs revealed that the charge of the sorting sequence did not influence the delta psi-dependence of translocation, suggesting a non-electrophoretic effect. These results suggest a contribution of the sorting sequence to the import driving mechanism in a manner that is not connected to the two known roles of the membrane potential. This assumption is supported by a preprotein with a charge-neutral amino acid exchange in

the hydrophobic segment of the sorting sequence, showing an import with an even lower delta psi-dependence than the wild type protein. We conclude that the sorting sequence of cytochrome b₂ strongly influences the delta psi-dependence of mitochondrial protein import by contributing to the driving mechanism in a novel charge- and localisation independent manner.

17. The Mammalian Homolog of the Yeast Sec Complex Subunit Sec63p: Part of a Multifunctional Transport Complex?

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Posttranslational protein transport into the yeast endoplasmic reticulum is mediated by the so called Sec-complex in the ER-membrane, containing a putative signal peptide receptor subcomplex (comprising Sec62p, Sec72p, Sec71p), a Hsp40 type membrane protein, called Sec63p, a protein conducting channel (the Sec61p-complex) plus luminal Hsp70s. Recently, human homologs of the yeast proteins Sec62p and Sec63p were discovered. We determined the concentrations of these two membrane proteins in dog pancreas microsomes and observed that these mammalian homologs are abundant proteins, present in almost equimolar concentrations as compared to the protein conducting channel subunit Sec61alphap. We suggest that Sec62p and Sec63p are parts of a multifunctional transport complex, prepared to function in posttranslational and cotranslational protein transport into as well as protein transport out of the mammalian endoplasmic reticulum.

Immunoprecipitation and purification experiments using microsomal extracts show, that both homologs form a complex with the Sec61p-complex. Furthermore the J-domain of Sec63p interact with the Hsp 70 homolog BiP. We found that the ATPase activity of BiP can be stimulated in a concentration dependent manner by GST-Sec63-J (recombinant J-Domain of Sec63p) and purified recombinant BiP binds to immobilized GST-Sec63-J in the presence of ATP with KD of 5x10-6M. For the luminal Hsp70s BiP and GRP170 it had been shown that they can stimulate cotranslational transport and for BiP to seal non-translocating protein conducting channels. Thus the interaction of Sec63p with the luminal Hsps could raise transport efficiency by directing the transfer of the nascent chain to the lumen or both proteins might be involved in the gating of the translocons. The observations that protein export into the cytosol, delivering proteins to the proteasome for degradation, depends on Sec61alphap and BiP in mammals and on Sec63p in yeast further support our suggestion of Sec63p being a part of a multifunctional transport complex.

18. Component Organization of the Machinery for the Thylakoidal Delta pH/Tat Pathway

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The Delta pH pathway is one of several distinct systems for protein translocation into the thylakoids of plant chloroplasts. This system exhibits several novel characteristics that include a consensus twin-arginine motif in the signal peptide of its substrates, the lack of soluble factor and NTP requirements, and the sole reliance on a transmembrane pH gradient for transport. In addition, the Delta pH pathway and the related bacterial Tat pathway can transport folded polypeptides. Two components of the Delta pH pathway translocon, Hcf106 and Tha4, have been genetically identified. Three components of the *E. coli* Tat pathway have been identified. TatB is the Hcf106 ortholog, TatA/E is the Tha4 ortholog, and TatC is a multispanning membrane protein. Recently we provided biochemical evidence for the role of Hcf106 and Tha4 in thylakoid protein transport. Here we report that a chloroplast TatC ortholog is required for the Delta pH pathway and describe the organization of components in the membrane. Arabidopsis genomic DNA homologous to TatC was used to isolate a pea chloroplast TatC cDNA. Antibody to the expressed cpTatC amino terminus, when prebound to thylakoids, specifically inhibited protein transport on the Delta pH pathway, but not the Sec or SRP pathways. Blue native gels were used to analyze organization of the components in the membrane. Anti-cpTatC reacted with a 700 kDa complex that was recognized with anti-Hcf106, but not anti-Tha4. Hcf106 also migrated as a 130 kDa species. Tha4 migrated as a 100 kDa species that did not contain Hcf106 or cpTatC. These results were supported by immunoprecipitation studies. Anti-cpTatC co-immunoprecipitated a fraction of Hcf106 and anti-Hcf106 co-immunoprecipitated all of the cpTatC. Tha4 was immunoprecipitated with anti-Tha4, but not with anti-Hcf106 or anti-cpTatC. None of these proteins was co-immunoprecipitated with anti-cpSecY or anti-cpOxa1p, which serve as translocon components of two other thylakoid translocation pathways. These results provide evidence that Hcf106, Tha4, and cpTatC all directly participate in protein transport on the Delta pH pathway. In addition, they suggest a model in which the cpTatC-Hcf106 complex assembles with Tha4 upon establishment of transport conditions to form an active translocon. The exact composition of the translocon may be determined by the size of the protein being translocated. This work was supported by an NIH grant to K.C.

19. Steric Chaperone-Induced Folding of Proteins Secreted by the Type II Secretion Pathway

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Exoproteins that use the type II secretion pathway in Gram-negative bacteria acquire their native structure in the periplasm. Correct folding is indispensable for secretion,

since exoproteins that fail to fold are not secreted but rapidly degraded in the periplasm. This folding is catalyzed by general folding catalysts, such as DsbA, and in addition, specific chaperones can be involved in this process. These specific chaperones can either be covalently linked to their substrates, such as the propeptides of many bacterial proteases, or exist as separate polypeptides, for example the lipase-specific foldases (Lifs) of many *Pseudomonas* and *Burkholderia* spp. Molecular chaperones generally function in the folding process by preventing off-pathway reactions, such as aggregation. In contrast, the propeptides of proteases directly catalyze the folding process by lowering a high-energy barrier on the folding pathway. Therefore, they have been designated "steric chaperones", since they contribute essential steric information, which is apparently lacking in the mature domain of the protein¹. The goal of the present study was to identify the mode of action of the Lifs. The role of the Lif from *Burkholderia glumae* in the folding of the cognate lipase was studied *in vitro* using purified proteins. In the absence of the Lif, unfolded lipase was found to refold into an intermediate with a similar secondary structure as the native lipase. However, this intermediate did not show any lipase activity. The intermediate could be converted into an active lipase upon addition of the Lif. This result demonstrates that the function of the Lif is not to prevent off-pathway folding reactions, but that it acts as a steric chaperone. Furthermore, interactions between the native lipase and the Lif were revealed in protease-protection assays and by affinity chromatography, consistent with a role of the Lif late in the folding pathway. The Lif- and propeptide-stimulated folding pathways of lipases and proteases, respectively, are thermodynamically not favorable². However, the proteins are locked in their native conformation because of the high transition energy for unfolding. This type of folding is favorable for proteins that have to survive in a harsh environment rich in proteases.

References: 1) Ellis, R. J. (1998) Trends Biochem. Sci. 23, 43-45. 2) Sauter, N. K., et al. (1998) Nature Struct. Biol. 5, 945-950.

20. Site-Specific Phosphorylation Used as a Marker for Membrane Protein Topology and as a Timer to Estimate the Time-Course of SRP-Dependent Protein Targeting to the Endoplasmic Reticulum

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We have developed a novel procedure to determine *in vivo* the cytosolic localization of membrane protein sequences by introducing a heptapeptide consensus sequence for specific phosphorylation by cAMP-dependent protein kinase. This phosphorylation tag could furthermore be used to assess the dynamics of protein targeting and translocation. The phosphorylation site was positioned at the N-terminus of a type III (Nexo/Ccyt) membrane protein which is only transiently exposed to the cytosol prior to its translocation into the lumen of the endoplasmic reticulum (ER). We found that the degree of phosphorylation increased with increasing length of the spacer between the phosphorylation site and the internal signal-anchor sequence from 20 up to 80 amino acids, reflecting the time the tag was exposed to the cytosol before

reaching the ER lumen. Extrapolation of the phosphorylation intensity to a spacer of zero length gives an estimate of the time required for SRP binding and targeting to the ER membrane. The results show that this process is quite rapid compared to the rate of translation, suggesting that — at least for the signal sequence tested — translation arrest is not necessary to allow targeting.

21. The *Haemophilus influenzae* DNA Processing Gene, *dprA*⁺, is not Required for the Translocation of Donor DNA out of the Transformasome

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Haemophilus influenzae is one of more than forty bacterial species capable of undergoing natural genetic transformation. The transformation process involves DNA binding to the competent cell surface, DNA translocation, targeting, and recombination. Previously, a mutation in the DNA processing gene, *dprA*⁺, was found to dramatically reduce the capacity of *H. influenzae* to undergo genetic transformation. Two mutant strains, GBH37F (*dprA*:mini-Tn10kan) and GBH41 (*dprA*⁺ Δ) were prepared to more precisely define the role that DprA plays in the transformation process. To determine whether DprA is involved in the translocation of DNA during transformation, we developed a PCR-based approach for the preparation of a uniformly labeled DNA substrate to be implemented in the nucleoside release assay. This assay permits the detection of radiolabeled deoxyribonucleosides as an indicator of donor DNA processing during the transformation event. Normally, donor DNA undergoes limited enzymatic degradation as it is prepared to potentially recombine with the recipient chromosome. The material released during processing is either randomly reincorporated into the chromosome or released into the cellular growth medium. As biochemical reference points for DNA processing, in addition to wild-type, *rec2* and *recA* (*rec1*) mutant strains were also analyzed in this study. Our results indicate that DprA is not required for the translocation of DNA into the cytosol; therefore, it must be involved in a more downstream process, such as the targeting of donor DNA to its homologous sequences in the chromosome, or the actual recombination of donor DNA with the recipient chromosome.

22. Establishment of a Translocation Intermediate on the Delta pH Pathway of Thylakoid Protein Translocation

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A subpopulation of nuclear encoded thylakoid proteins are translocated to the thylakoid lumen via the Delta pH translocation machinery. Genetic and biochemical evidence has shown that the Delta pH pathway components are evolutionarily related to the bacterial Tat protein transport system which can translocate folded apoprotein-cofactor conjugates. To facilitate biochemical

investigation of the thylakoid Delta pH pathway of protein translocation, we undertook the development of a translocation intermediate. Coding sequences for recombinant proteins were constructed by PCR-based methods. Chloroplasts were isolated from *Pisum sativum*. *In vitro* translation products were radiolabeled and the results of assays were visualized by fluorography of SDS-PAGE gels. Here we demonstrate the achievement of protein translocation arrest on the Delta pH pathway. A recombinant construct consisting of pOE17, a ninety amino acid linker and three IgG binding domains of Protein A is processed to mature form in a transport dependent fashion. Protease treatment of the arrested protein leaves a protected fragment slightly larger than mOE17 inside the lumen, while the Protein A moiety is degraded on the cis side of the membrane. The establishment of a translocation intermediate arrested while traversing the membrane in a N- to C- terminus orientation argues for the use of a channel by the Delta pH pathway and is an essential step in the investigation of its components and their mechanisms of activity.

23. Integration Requirements for Albino3, an Oxa1p Homolog, Suggest a Novel Chloroplast Protein Transport Pathway

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The mitochondrial inner membrane protein, Oxa1p, has been shown to participate in its own integration into the membrane, likely by allowing its N-terminal tail to be exported out of the matrix. Its thylakoid homolog, Albino3 (ALB3), has also proven to be part of a translocon which integrates LHCP. The goal of our research is to ascertain whether ALB3 similarly uses itself for integration or follows one of the four known localization pathways that exhibit distinct energetic and protein requirements. Stromal proteins and a specific nucleotide are required for proteins using both the Sec and SRP transport pathways, while the Delta pH (TAT) pathway requires only a trans-thylakoid pH gradient. Other proteins enter the thylakoid membrane through a spontaneous pathway that is defined by the lack of a requirement for protein, proton motive force (PMF), or nucleotide. The necessity of these factors was examined in assays that reconstitute Alb3 integration into isolated thylakoids. ALB3 integration was most efficient in the presence of stroma, ATP, and a PMF. Assays conducted in the absence of stromal proteins showed a marked decrease in the amount of ALB3 integrated. This decrease recurred in assays that included apyrase to scavenge ATP and GTP. The nucleotide analog AMP-PNP mimicked the influence of apyrase, whereas GMP-PNP had no affect. A requirement for the chloroplastic SRP or SecA was ruled out by competition and the use of stromal fractions enriched in these proteins. Dissipation of the PMF with the protonophore, nigericin, did not abolish ALB3 transport as it does TAT pathway substrates, but did suggest an enhancing effect of PMF on transport. Finally, antibodies to the recognized translocon components ALB3, Hcf106, Tha4, and cpSecY, were tested for their ability to forestall localization of ALB3

and identified pathway substrates; however, none of these were successful in preventing ALB3 integration. These data demonstrate that ALB3 is integrated into the thylakoid membrane by a previously undefined pathway which utilizes an undetermined stromal factor and ATP.

24. The Physiological Basis for the Conservation of the Signal Recognition Particle Targeting Pathway in *E. coli*

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The *E. coli* signal recognition particle (SRP) is an essential ribonucleoprotein complex that targets inner membrane proteins (IMPs) to membrane-bound translocons. Since SRP depletion only partially inhibits IMP insertion, however, it is not clear why the particle is critical for cell survival. Insights into this question emerged from experiments in which we analyzed the physiological consequences of reducing the intracellular concentration of SRP below the wild-type level. We found that even moderate SRP deficiencies, which have little effect on cell growth, led to the induction of a heat shock response. Genetic manipulations that suppress the ability of cells to mount a heat shock response were synthetically lethal with moderate SRP deficiencies, indicating that the elevated production of heat shock proteins is required for cell viability. Although it is conceivable that the heat shock response serves to increase the level of molecular chaperones that target IMPs via SRP-independent mechanisms, SRP-deficient cells did not show an increased dependence on either GroEL/ES or DnaK/J. By contrast, the viability of SRP-deficient cells was highly dependent on the presence of heat shock-regulated proteases. Moreover, a model IMP that was mislocalized in the cytoplasm as the result of SRP depletion was rapidly degraded in control cells but was hyperstabilized in a strain that contains multiple protease mutations. These results provide direct evidence that the heat shock response protects SRP-deficient cells by increasing their capacity to degrade mislocalized IMPs and suggest that SRP has been conserved in prokaryotes in part to prevent protein aggregation in the cytoplasm by maximizing the efficiency of IMP insertion.

25. The Energetic Requirements of Protein Transport via the Delta pH Dependent/Tat Translocation Pathway in Thylakoids

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The Delta pH-dependent (Tat) protein translocation pathway of thylakoids uses a transmembrane proton gradient as the sole energy source to power the import of nuclear-encoded proteins into the thylakoid lumen. To date, direct utilization of the proton gradient by the Tat pathway machinery has not been shown, nor has an energetic barrier required for protein transport been detected. The goal of this investigation is to directly

measure both of these parameters to obtain a measure of the free energy of protein translocation on the Tat pathway, quantified as the product of the minimum electrochemical proton potential required for transport and the proton utilization per protein translocated stoichiometry. Determination of the energetic threshold required for protein transport was made by conducting protein import assays while concurrently monitoring the delta pH in a suspension of isolated thylakoids. Substrate-specific threshold delta pH values were detected, below which no protein transport occurs, and above which the rate of protein transport increases linearly. For the substrates pOE17 and pOE23, measured threshold DpH values were 0.84 and 1.97 pH units, respectively (corresponding 5.70 and 12.19 kJ per mol proton utilized). Evidence for direct proton gradient consumption by the Tat pathway machinery has been obtained by monitoring decreases in the measured steady-state delta pH upon injection of saturating concentrations of substrate into thylakoid suspensions under limiting light conditions. These data are supported by an observed increase in dark transient proton efflux rates for isolated thylakoids in the presence of precursor substrates. Assays designed to independently measure the free energy cost of Tat protein translocation by monitoring increases in ATP hydrolysis or decreases in ATP synthesis attributable to protein import are described.

26. Peroxisome Synthesis in the Absence of Pre-existing Peroxisomes

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The peroxisome is a single-membrane bound organelle found in virtually all eukaryotic cells and contains enzymes involved in many metabolic processes. Although much is known about the import of peroxisome matrix proteins, including specific targeting signals, receptors, and translocation machinery, relatively little is known about the biogenesis of the peroxisome membrane and the targeting of peroxisome membrane proteins (PMPs). Defects in peroxisome biogenesis lead to a group of lethal diseases known as the peroxisome biogenesis disorders (PBD). In the vast majority of PBD cell lines, the defect could be assigned strictly to the matrix protein import pathway. However, 4 cell lines have been identified with the unusual cellular phenotype of an inability to import both peroxisome membrane and matrix proteins. Discovery of the gene defective in one of these cell lines (PBD399) has lead to the identification of a cytosolic PMP receptor, PEX19. Here we report the identification of the genes defective in the remaining 3 cell lines. PBD061 cells have an inactivating mutation in the PEX16 gene whereas PBD400 and PBD401 both have inactivating mutations in the PEX3 gene. Previous studies have suggested that peroxisomes always arise from pre-existing peroxisomes yet we find that expression of PEX3 or PEX16 in their deficient cell lines results in the formation of new peroxisomes. Previous studies in plants and yeast have also suggested that brefeldin A treatment inhibits PMP import yet we find that in humans neither the targeting nor the

function of PEX3 and PEX16 is inhibited by brefeldin A treatment. We propose that peroxisomes may form by either of two pathways, one that involves division of pre-existing peroxisomes and another that involves PEX3 and PEX16 in the formation of new peroxisomes.

27. Characterization of Microsomal Signal Peptide Processing Enzymes

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The signal sequence of preprolactin undergoes a minimum of two proteolytic processing events at the endoplasmic reticulum (ER) membrane. First the signal sequence is removed from preprolactin by the action of signal peptidase, generating the intact signal peptide (SP) and mature prolactin. The intact signal peptide is cleaved again to yield a lower molecular weight peptide (PSP) which is subsequently released to the cytosol. As a first step toward understanding the role(s) of both signal peptide processing activities in the transport of preproteins across the ER membrane, we have analyzed the sensitivities of both signal peptidase and microsomal signal peptide peptidase (MSPP) activities to various protease inhibitors. Cell-free translations supplemented with canine rough microsomes were used to generate truncated translocation intermediates of preprolactin which have initiated translocation but retain their signal sequences. Translation reactions were treated with 0.01% Sarkosyl to permeabilize membranes prior to treatment with various protease inhibitors. Inhibitors specific to each of the four general families of protease inhibitors were used, including pepstatin, α -2-macroglobulin, amastatin, antipain, leupeptin, elastatinal, phosphoramidon, phenyl-methyl-sulfonyl-fluoride and chymostatin. Puromycin treatment released the truncated preprolactin from the ribosome, thus synchronizing removal of the signal sequence and allowing its subsequent processing to be observed over time. Translation reactions were subjected to polyacrylamide gel electrophoresis and bands visualized by autoradiography or phosphorimaging. The generation of processed signal peptide from the intact cleaved signal peptide at 15 minutes after puromycin treatment was reduced 84% by chymostatin, 55% by antipain and 30% by leupeptin. The other inhibitors tested had no effect on signal peptide processing. Surprisingly, signal peptide removal was reduced by 75% following chymostatin treatment. These data indicate that mammalian signal peptidase and MSPP appear to belong to the serine- and cysteine-/serine- protease families, respectively. In this respect they are similar to their counterparts in *E. coli*. Identification of specific effective inhibitors of signal peptidase and MSPP will allow the study of the roles of these enzymes in protein transport across the ER membrane.

28. Two Different Types of Nuclear Import Carriers, p10/NTF2 and Karyopherin β -1, Compete for Docking Sites at the Nuclear Pore Complex During Nuclear Import

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Purpose: The protein p10/NTF2 is known to mediate the nuclear uptake of RanGDP, but whether it may have additional roles in the cell is unknown. Also unclear is the mechanism by which any nuclear transport complex actually moves through the nuclear pore complex (NPC). This movement is thought to involve repeated association/dissociation reactions with sequential NPC proteins, but whether all carriers use the same or different "tracks" through the NPC is unknown. **Methods and Materials:** A number of p10 point mutants were generated and tested for their ability to bind Ran, their cellular localization and activity *in vivo*, for their ability to directly mediate the nuclear import of FITC-RanGDP, and for their effects on the import of a classical nuclear localization sequence containing substrate (NLS-BSA). We also analyzed the binding properties of p10 (wt and mutants) and karyopherin β -1 to various NPC proteins. **Results:** Of the p10 mutants, only the D23A mutant retained the ability to bind both Ran and the nucleoporins. In addition, this mutant was also competent to mediate the nuclear import of RanGDP *in vitro*. Strikingly, however, D23A p10 inhibited the simultaneous nuclear import of NLS-BSA mediated by karyopherins α and β -1. The D23A p10 was found to have a higher affinity for the NPC than wt p10 and able to compete much more efficiently with karyopherin β -1 for docking sites at the NPC than wt p10. **Conclusions:** We conclude that two different types of nuclear import carriers (p10 and karyopherin β -1) use at least some of the same docking sites during their respective passages through the NPC and that D23A p10 is able to inhibit NLS-BSA import by occupying vital docking sites at the NPC that are also needed by karyopherin β -1 for classical NLS-mediated import. Further biochemical studies on the nature of p10 and karyopherin β -1 binding to the NPC are in progress.

29. Selectivity in Sorting of Preassembled Cytosolic PTS1-Proteins in the Yeast *Hansenula polymorpha*

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Peroxisomes are organelles found in all eukaryotic organisms studied so far. Unlike other cellular organelles, their function may be highly diverse, dependent on cell-type and external conditions it encounters. In human, for instance, they are involved in a great variety of anabolic and catabolic pathways, including plasmalogen and cholesterol biosynthesis as well as β -oxidation of very-long chain fatty acids. Peroxisome malfunctioning causes severe inherited disorders, such as Zellweger Syndrome. Peroxisomal matrix proteins are post-translationally sorted from the cytosol. Two targeting signals for peroxisomal matrix proteins have been described; PTS1, a C-terminal tripeptide (SKL and variants) and PTS2, an N-terminal nonapeptide (-RL-X₅-H/QL). Both PTS1 and PTS2

proteins can be imported as folded oligomers. In *Hansenula polymorpha* peroxisomes are massively induced upon growth of this yeast in methanol-containing media. Alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT) reside in these peroxisomes and catalyze the initial steps in methanol-metabolism. All three enzymes are PTS1-proteins and are active as homooligomers (AO, octamer; DHAS, dimer; CAT, tetramer). In spite of the fact that folded proteins can be imported into peroxisomes, we found that alcohol oxidase, when assembled in the cytosol, was not incorporated into newly-formed peroxisomes after a shift of a temperature sensitive mutant of *H. polymorpha*, *pex1-6^{ts}* (here renamed *pex1-6^{ts}*), defective in peroxisome biogenesis (Waterham *et al.*, 1993). To determine whether this is a general feature for peroxisomal matrix proteins in *H. polymorpha*, we expressed a PTS1-tagged Green Fluorescent Protein in *H. polymorpha pex1-6^{ts}* cells under control of the alcohol oxidase promoter. We found that, in contrast to AO, cytosolic GFP-SKL and also endogenous DHAS were sorted to newly formed peroxisomes at permissive conditions. In addition, reappearance of peroxisomes at permissive temperature also occurred when the cells were grown in the presence of cycloheximide. These data show that 1) assembled alcohol oxidase is not competent for import into peroxisomes, whereas dihydroxyacetone synthase and GFP-SKL are, and 2) after a shift of *pex1-6^{ts}* cells to permissive conditions, peroxisomes can be assembled from pre-existing proteins, even though no peroxisomal remnants are detected at restrictive conditions.

30. Identification of the Peroxisomal Sorting Determinants of the Mammalian Integral Membrane Proteins PMP22 and PMP24

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In mammals, peroxisomal integral membrane proteins (PMPs) appear to be synthesized in the cytosol and inserted into the peroxisome membrane post-translationally. While considerable progress has been made in peroxisomal matrix protein import, it is not well understood how the solubility of PMPs in the cytoplasm is maintained and how these molecules recognize their target membranes. Until now, the sorting determinants (mPTSSs) of four integral PMPs have been defined [for references, see 1]. All these mPTSSs contain patches of positively charged amino acids and are thought to be localized to the matrix side of the peroxisome membrane. In two cases the mPTSSs require, in addition to the luminal portion of the protein, a transmembrane domain to be functional [1]. However, before any solid conclusions about consensus sequences or common structural properties can be drawn, it is necessary to identify additional mPTSSs. In this context, we constructed a number of plasmids encoding chimeric proteins composed of GFP and portions of rat PMP22 or human PMP24, two peroxisomal integral membrane proteins of which the function still remains to

be elucidated. These constructs were expressed in Chinese hamster ovary (CHO) cells and the sorting behaviors of the corresponding chimeric proteins were analyzed. In addition, in view of the recent observations that Pex19p binds (the mPTSS of) a broad spectrum of newly synthesized PMPs and facilitates their insertion into the peroxisome membrane [2], we investigated whether the delineated peroxisomal sorting signals of rat PMP22 and human PMP24 retained an interaction with human Pex19p. **References:** [1] Koller *et al.* J Cell Biol 146;1999: 99-112. [2] Sacksteder *et al.* J Cell Biol 148;2000: 931-944. **Acknowledgements:** M.F. is a post-doctoral fellow from the 'Fonds voor Wetenschappelijk Onderzoek - Vlaanderen'.

31. Identification, Cloning and Targeting of a Putative Human Peroxisomal 2,4-Dienoyl-CoA Reductase

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Recently, the use of the gVI-cDNA phage display technology to clone cDNAs coding for peroxisomal enzymes by affinity selection using immobilized antisera directed against peroxisomal subfractions was reported by some of us [1]. Here we describe the isolation and cloning of a human cDNA coding for a novel peroxisomal 2,4-dienoyl-CoA reductase related protein using this technique. The obtained human cDNA contains an open reading frame of 909 bases, encoding a protein of 303 amino acids with a calculated molecular mass of 32.6 kDa. Primary structure analysis revealed that this protein is a member of the 2,4-dienoyl-CoA reductase (DCR) family. The amino acid identity with the rat 2,4-dienoyl-CoA reductase is 72% (accession no. AAF14047). However, using either 2-trans,4-trans-hexadienoyl-CoA or 2-trans,4-trans-decadienoyl-CoA as a substrate, no DCR activity was observed with the bacterially expressed biotin tagged protein. The C-terminal tripeptide of the putative DCR is AKL, a possible peroxisome targeting signal 1 (PTS1). Most enzymes present in the matrix of peroxisomes contain a PTS1 and are recognized by the Pex5 protein (Pex5p) after which they are imported into peroxisomes [2]. The recombinant protein was recognized by the human PTS1-receptor (Pex5p) in a ligand blot overlay assay and in a microtiter plate assay. Binding was competitively inhibited by a PTS1-containing peptide and was abolished when the last amino acid of the PTS1 signal was deleted. Transfection of CHO-cells with plasmids coding for green fluorescent protein (GFP)-DCR fusions confirmed the functionality of the PTS1. **References:** [1] Fransen *et al.* Biochem J 340;1999: 561-568. [2] Hettema *et al.* Biochim Biophys Acta 1451;1999: 17-34. **Acknowledgements:** L.A. is supported by a fellowship from the "IWT-Vlaanderen", M.F. by a post-doctoral fellowship from the "FWO-Vlaanderen".

32. Studies on the Targeting Signals of Peroxisomal Integral Membrane Peroxins

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The correct and functional assembly of peroxisomes requires coordinated interactions between so called peroxisome assembly proteins (peroxins, abbreviated Pexp). Hitherto, 23 peroxins have been identified [1]. Knowledge of the

peroxisomal targeting of peroxisomal integral membrane proteins (PMPs) remains scanty and confusing. The few sequences that have been reported to be involved in the targeting of PMPs (mPTSs) show very little sequence homology [for references see 2]. Recently we cloned HsPMP34 [3], the human counterpart of *Candida boidinii* PMP47, and started to analyze its targeting by means of transfection studies with constructs coding for Green Fluorescent Protein (GFP)-HsPMP34 fusion proteins. The sequence corresponding to the mPTS of CbPMP47 appeared to be necessary but not sufficient for targeting of HsPMP34 to the peroxisomes. The delimited targeting domain is much larger, starting from before the second membrane span and ending just before the fifth membrane span. In a similar approach, the topogenic domains of several human integral membrane peroxins were delineated. In the case of HsPex2p, HsPex10p, HsPex11p'alpha', HsPex11p'beta', HsPex12p and HsPex16p, the delimited mPTSs cover approximately half of the total protein and contain one membrane spanning domain. This is different from the defined mPTS of HsPex3p, which covers the 40 N-terminal amino acids of the molecule [4]. **References:** [1] Brown et al. Mol Biol Cell 11;2000: 141-152. [2] Koller et al. J Cell Biol 146;1999: 99-112. [3] Wylin et al. Eur J Biochem 258;1998: 332-338. [4] Kammerer et al. FEBS Lett 429;1998: 53-60. **Acknowledgements:** M.F. is supported by a fellowship from the 'FWO-Vlaanderen'.

33. Delineation of Three Functional Domains of Human Pex5p

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Pex5p, the import receptor for peroxisomal matrix proteins containing a type-1 peroxisomal targeting signal, is probably the best characterized peroxin. In general, mutants defective in Pex5p are impaired in the import of PTS1, but not PTS2 matrix proteins. However, in mammals, two functionally distinct isoforms of the PTS1 receptor are generated by alternative splicing of a single exon. The shorter form Pex5pS mediates only PTS1 import. The longer form Pex5pL is required for peroxisomal import of PTS1 and PTS2 proteins [for references see 1]. Available evidence now points to a model in which Pex5p binds with high affinity to newly synthesized PTS1 proteins in the cytosol, "cycles" onto the peroxisome membrane, releases its cargo and "recycles" back to the cytosol [2]. Because little is known about the factors and mechanisms that direct the Pex5p-receptor-ligand complex to the peroxisomal membrane and disassemble this complex once it is docked to the peroxisome, we delineated the domains of human Pex5p responsible for binding to its already identified interaction partners PTS1 [3,4,5], Pex14p [6,7] and Pex12p [8]. Our results demonstrate that (i) the TPR domains of Pex5p are required for binding to PTS1 and Pex12p, but not for binding to Pex14p, (ii) the binding domains of Pex5p for PTS1 and Pex12p do overlap but are not identical, (iii)

binding of Pex5p to Pex12p requires the functional RING finger motif (C_3HC_4) of Pex12p and (iv) the *in vitro* and *in vivo* approaches used to delineate the Pex14p binding domain of Pex5p give rise to different, even conflicting, conclusions.

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Acknowledgements: M.F. is a post-doctoral fellow from the 'Fonds voor Wetenschappelijk Onderzoek - Vlaanderen'. L.A. is supported by a fellowship from the Flemish 'Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie, IWT'.

34. A Microsomal GTPase is Required for Glycopeptide Export from Mammalian Endoplasmic Reticulum

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Bidirectional transport of proteins *via* the Sec61p translocon across the endoplasmic reticulum membrane is a recognized component of the ER quality control machinery. Following translocation and engagement by the luminal quality control system, misfolded and unassembled proteins are exported from the ER lumen back to the cytosol for degradation by the proteasome. Additionally, other ER contents, including oligosaccharides, oligopeptides and glycopeptides are efficiently exported from mammalian and yeast systems indicating that bidirectional transport across ER membranes is a general eukaryotic phenomenon. Glycopeptide and protein export from ER in *in vitro* systems is both ATP and cytosol-dependent. Using a well established system to study glycopeptide export from eukaryotic ER membranes (Romisch, K. & B.R.S. Ali, 1997, *Proc. Natl. Acad. Sci. USA*, 94: 6730-6734) and conventional liquid chromatography, we isolated a single polypeptide species of 23 kDa from rat liver cytosol that was capable of fully supporting glycopeptide export from rat microsomes in the presence of an ATP-regenerating system. The protein was identified by mass spectrometric sequence analysis as guanylate kinase, a housekeeping enzyme critical in the regulation of cellular GTP levels. We confirmed the ability of GK to substitute for complete cytosol by reconstitution of glycopeptide export from rat liver microsomes using highly purified recombinant GK from *Saccharomyces cerevisiae*. Most significantly, we found that GK, and hence cytosolic components, requirement was fully bypassed by low micromolar concentrations of GDP or GTP. Similarly, export was inhibited by nonhydrolysable analogues of GDP or GTP indicating a requirement for GTP hydrolysis. Membrane integrity was fully maintained under assay conditions as no luminal ER proteins were released. Competence for glycopeptide export was abolished by very mild trypsin treatment of microsomes indicating the presence of an essential protein on the cytosolic face of the ER

membrane. These data demonstrate that glycopeptide export from rat liver microsomes is controlled by a microsomal GTPase and is independent of cytosolic protein factors.

35. The Effects of Charge-Pair Interactions on a Transmembrane Helix in the Microsomal Membrane

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We have examined the effects of charge-pair interactions on the conformation and position of a TM helix in the lipid bilayer using the "glycosylation mapping" technique (Nilsson et al., 1998). This technique was previously developed in our laboratory, and it allows an accurate measurement of the position of a TM helix in the ER membrane using the active site of the ER enzyme oligosaccharyl transferase as a reference point. We made a set of constructs containing pairs of positively and negatively charged residues (Lys and Asp, respectively) in various positions in a model TM helix composed of 23 Leu residues and analyzed the results by glycosylation mapping. A large shift of the position of the transmembrane helix can be observed when Lys and Asp are placed in a close proximity in the same helix. This shift was reversed when Asp and Lys are placed in the (i, i+3) and (i, i+4) positions. The results show clear evidence of salt-bridge formation between residues located one helical turn apart within the TM helix.

36. Isolation and Characterization of *Escherichia coli* Signal Recognition Particle and Secretion Mutants that are Defective in Membrane Protein Assembly

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We carried out a genetic screen utilizing the MalF-LacZ fusion protein with the intention of finding new classes of mutants affecting the disulfide bond formation pathway. We had used this fusion previously to look for lac⁺ colonies of *E. coli* strain expressing this fusion protein and had successfully isolated *dsbA* and *dsbB* phenotypic null alleles. These mutants had inactivated the disulfide bonds presumably formed in the β-galactosidase of the MalF-LacZ fusion protein, and in doing so allowed the retraction of the partially exported beta-galactosidase to the cytoplasm where it is functional. The new screen looked for weaker mutants than the ones previously found by seeking partial restoration of β-galactosidase activity to the fusion protein. The screen generated mainly two classes of mutants: those affecting the formation of disulfide bond and those that did not. The first class includes 27 *dsbA* mutants and 20 *dsbB* mutants. The *dsbA* mutants include three novel amino acid substitutions and the *dsbB* mutants include two such new changes. The second class of mutants that did not affect disulfide bond formation pathway includes mutations in the signal recognition particle (SRP) and the Sec machinery, both important for another cellular process, membrane protein insertion. The SRP mutants include mutations in 4.5S RNA, Ffh, and the

SRP receptor FtsY. The Sec mutants include mutations in the *secA* operon. By attaching a biotinylatable domain to the periplasmic region of a membrane protein and looking at the biotinylation state of the fusion protein, we examined *in vivo* in growing cells whether these mutants are defective in the process of membrane protein insertion since biotinylation only occurs in the cytoplasm. We show that both the SRP mutants and the Sec mutants are defective in this process. They differ, however, in that while the Sec mutants also show a secretion defect, the SRP mutants did not. We next examined the 4.5S RNA mutants and found that their mature form 4.5S RNA levels were lowered. In addition, two 4.5S RNA mutant alleles also have another 4.5S RNA species larger than the mature form, presumably representing the unprocessed 4.5S RNA.

37. The Fate of Endoplasmic Reticulum-Bound Ribosomes Following the Termination of Protein Translation — is Ribosome Exchange Coupled to Protein Translocation?

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The current model for cotranslational translocation in the mammalian endoplasmic reticulum (ER) identifies the termination of protein synthesis as the signal for ribosome release from the ER membrane to the cytosol. Such a ribosome release event has been demonstrated to regulate the activity of translocation channels by serving as a signal for channel closure. At present, however, the physiological mechanism for ribosome release remains largely unexplored. We have initiated a series of biochemical and morphological studies of ribosome exchange in a rat liver cell culture system and report that membrane-bound ribosomal subunits remain in stable association with the ER membrane following the termination of protein synthesis. In studies with pactamycin, an inhibitor of the initiation reactions of protein synthesis, and puromycin, which elicits premature termination, it was found that following physiological termination (as observed in the presence of pactamycin) or artificial termination (as observed in the presence of puromycin), the vast majority of ribosomes remained membrane associated. Preliminary characterization of the subunit structure of the post-termination membrane-bound ribosomes indicates that small subunits are released to the cytosol whereas large subunits are retained on the ER membrane. These results suggest that ribosome release from the ER membrane is regulated independently of protein translation and translocation. Supported by DK47897 (CVN).

38. Characterization and Subcellular Localization of the Peroxisomal Membrane Protein, PMP50

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Evidence suggests that nascent vesicles of ER origin may be necessary for the formation of functional peroxisomes. The recently identified 50 kDa membrane-associated protein, termed PMP50, is the only mammalian peroxisomal protein known to be synthesized on membrane-bound polysomes. PMP50 co-sedimented with peroxisomal membranes and pulse-chase experiments indicated that it first associated with the ER and then accumulates with peroxisomes. Here we show that upon microinjection of alcohol oxidase, a yeast

peroxisomal matrix protein, mammalian cells exhibit a punctate staining pattern specific for PMP50 but these vesicles do not contain catalase, a bona-fide peroxisomal marker. Database searches indicate strong homology between PMP50, the vesicular transport protein ERGIC-53, and the Galectins, a family of proteins with galactose binding signatures. Based on previous work and this sequence similarity, we hypothesized that PMP50 may act as a shuttle between the ER and peroxisomes. Indeed, in yeast, some peroxisomal proteins were demonstrated to be glycosylated. Galectins have an affinity for β -galactose sugars. To examine the binding specificity of PMP50, we have microinjected sugars into the cytosol of rat fibroblasts transfected with a full-length HA-tagged version of PMP50. We found that upon the microinjection of lactose, but not sucrose, PMP50 exhibits a cytoplasmic staining pattern, indicating that PMP50 has a stronger affinity for lactose and therefore may bind preferentially to lactose-containing molecules. In order to further study the subcellular localization of PMP50, we have constructed full length and C-terminal HA-tagged versions of PMP50 and have produced a stable cell line. We have examined the association of PMP50 with peroxisomes. Digitonin, salt and Triton X-114 phase separation experiments, followed by western blot analysis revealed that PMP50 is found almost exclusively in membrane fractions. Immunofluorescence studies showed that PMP50 exhibits a vesicular staining pattern and some, but not all of these vesicles colocalize with the peroxisomal markers PMP70 and thiolase. Transient transfections of truncated versions of PMP50-HA showed that the C-terminal end (AA107-323) of this hybrid protein binds to vesicles indicative of peroxisomes. We propose that PMP50 may be directly involved in peroxisome biogenesis by participating in the shuttling of cargo and membrane components of newly formed peroxisomes.

39. Formation of Peroxisomes from PMP50-Containing Preperoxisomal Vesicles

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Most complementation groups of mammalian cells genetically deficient in peroxisome biogenesis contain abnormal membrane structures known as peroxisome ghosts. These structures are devoid of peroxisomal matrix proteins but contain the integral peroxisomal membrane protein PMP70. Upon genetic complementation these mutants regain the ability to incorporate matrix proteins. Recent studies have demonstrated that these ghosts are precursors for mature peroxisomes in pex5-mutant cells. The pex16-mutant cells do not exhibit PMP70-containing vesicles. However, complementation of these pex16-mutant cells implies that a preperoxisomal vesicle must exist. It is postulated that the integration of PEX16 into preperoxisomes creates nascent peroxisomal structure. However, there has been no evidence for the presence of such preperoxisomal structures in pex16-mutant cells so far. Here we show that human cells mutated at PEX16 target the peroxisomal membrane protein PMP50 to

vesicles upon transfection of a PMP50-EYFP expression plasmid and that these PMP50-containing vesicles are destined to become mature peroxisomes. Pex1-mutant cells do contain peroxisomal ghosts and the protein PMP70 is visualized on membrane structures in these cells. We have performed fusion experiments between pex16-mutants cells expressing a PMP50-EYFP hybrid protein and pex1-mutant cells. We observed that in cells fused in the presence of cycloheximide, some PMP50-EYFP colocalized with PMP70 to vesicular structures. Moreover, we show that these vesicles become import competent since the peroxisome matrix protein catalase is localized to vesicles in the fused cells only. Therefore, we demonstrated that both, the PMP50-containing vesicles of the pex16-mutant cells and the PMP70-containing ghosts from the pex1-mutant cells are precursors of mature peroxisomes. These two import incompetent vesicles may fuse together to create an import competent peroxisome. In conclusion, our findings demonstrate the requirement of primary vesicles containing PMP50 for the maturation of peroxisomes. Moreover, PMP50 seems to act upstream of PEX16 in the process of peroxisome biogenesis. We are investigating as well a role for PMP50 in the process of vesicle formation.

40. Filamentous Phage f1 are Transported Across Membranes Through Phage-Encoded Aqueous Channels

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Purpose: Proteins can only cross the endoplasmic reticulum, mitochondrial, chloroplast or inner bacterial membranes as an unfolded linear polymer. However, many macromolecules fold, and even oligomerize, before they cross the outer bacterial membrane and they cross without compromising the integrity of the membrane. One such example is filamentous phage f1 which exits its *Escherichia coli* host as a 70A diameter cylinder. The phage-encoded protein pIV, which shares homology with many other proteins involved in toxin export across the outer membranes of *Salmonella*, *Shigella*, *Yersinia*, and *Vibrio cholerae*, is required for this transport. We examine how pIV transports phage without compromising the integrity of the bacterial membrane. Specifically, the goal is to test if pIV forms a transmembrane aqueous channel for the transport of filamentous phage. **Methods and Materials:** The potential membrane transport activity of pIV was examined both *in vitro* (purified and reconstituted into planar lipid bilayers) and *in situ* (by examining transport of large macromolecules through pIV in the living *E. coli*). **Results:** Purified and reconstituted pIV protein forms a large channel in lipid bilayers. The biophysical properties of the channel are affected by single point mutations in the pIV gene (demonstrating that the channel is due to pIV and not a co-purifying contaminant). In the intact bacteria, pIV increases the permeability of the outer membrane to macromolecules. Under normal physiological conditions the pIV channels are closed. However, point mutations that increase the probability of the channel opening in the *in vitro* electrophysiological assays also increase the probability of the channel opening *in situ*. Transport of macromolecules through these channels is blocked during phage export. **Conclusions:** pIV forms a large (>6 nm) channel in the outer membrane through which phage

are extruded. This is the first demonstration of such a channel in the type II and type III secretion systems in bacteria and the first demonstration that the channels are used for export.

41. PEX19 Binds Multiple Peroxisomal Membrane Proteins, is Predominantly Cytoplasmic, and is Required for Peroxisome Membrane Synthesis

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Peroxisomes are components of virtually all eukaryotic cells. While much is known about peroxisomal matrix protein import, our understanding of how peroxisomal membrane proteins (PMPs) are targeted and inserted into the peroxisome membrane is extremely limited. Here we show that PEX19 binds a broad spectrum of PMPs, displays saturable PMP binding, and interacts with regions of PMPs required for their targeting to peroxisomes. Furthermore, mislocalization of PEX19 to the nucleus leads to nuclear accumulation of newly synthesized PMPs. At steady-state, PEX19 is bimodally distributed between the cytoplasm and peroxisome, with most of the protein in the cytoplasm. We propose that PEX19 may bind newly synthesized PMPs and facilitate their insertion into the peroxisome membrane. This hypothesis is supported by the observation that loss of PEX19 results in degradation of PMPs and/or mislocalization of PMPs to the mitochondrion.

42. Characterization of the tRNA Import Pathway into the Mitochondrion of *Trypanosoma brucei*

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Although RNAs that function in mitochondria are generally encoded by the mitochondrial genome, all of the mitochondrial tRNAs of *Trypanosoma brucei* are nuclear encoded and subsequently imported into the mitochondrion. Three different classes of nuclear-encoded tRNAs have been identified: 1) cytosolically localized tRNAs, 2) mitochondrially localized tRNAs, and 3) tRNAs that are shared between both compartments. Precursor tRNAs, containing a 5' extension, were identified in the mitochondrion of *T. brucei*. An RNase P activity in mitochondrial extract was able to process these 5' extensions on the precursors to yield mature tRNAs. This suggested that at least some tRNAs are imported as precursors rather than as mature tRNAs. An *in vitro* import system was developed in *T. brucei* using isolated mitochondria from trypanosomes. The system uses synthetic tRNAs transcribed in tandem from a nuclear-encoded genomic cluster, tRNA^{Ser} and tRNA^{Leu} separated by a 59 nt intergenic region. The tandem tRNA imports rapidly, whereas, the mature tRNAs fail to import. The *in vitro* system shows a requirement for ATP, a protein factor associated with the mitochondrial membrane, and a membrane potential. Kammerer and Hajduk have proposed an import model where transcription of the individual tRNAs localizes them to the cytosol, and tandem

transcription of the tRNAs as one long precursor transcript localizes the tRNAs to the mitochondrion. This would involve a failure of RNA polymerase to terminate after tRNA^{Ser}. The 59 nt intergenic region was identified as the minimal cis element involved in localization to the mitochondrion by *in vitro* import analysis of 5' and 3' deletions on the tandem tRNA^{Ser/Leu}. An *in vivo* import system based on homologous recombination of tRNA^{Ser/Leu} into the nuclear genome was developed to further characterize the cis elements and trans factors involved in the tRNA import pathway, as well as to determine the mechanism of RNA polymerase III transcription that may play a role in directing cytosolic versus mitochondrial localization of tRNAs. A point mutation was made in the variable arm of tRNA^{Leu} of the tRNA^{Ser/Leu} cluster in order to distinguish the mutated tRNA from the endogenous tRNA population in the transfected trypanosomes by a poison primer extension assay. Currently, the primer extension assay detects the presence of the mutated tRNA^{Leu} in the mitochondria of transfected *T. brucei*. The mutated tRNA is not detected in wild type *T. brucei*.

43. Acidic Residues Accelerate the Processing of PsbX in the Thylakoid Membrane

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Hydrophobic proteins are inserted into the thylakoid membrane by two broad types of mechanism. A subset of proteins use an 'assisted' pathway, where insertion relies on signal recognition particle (SRP), FtsY, GTP and membrane-bound protein transport apparatus, whereas others insert by an apparently spontaneous pathway that does not require any known protein targeting apparatus or energy source. Surprisingly, most of the latter class are nevertheless synthesized with cleavable N-terminal signal peptides, and it has been proposed that these function by providing an additional hydrophobic region that aids insertion of the transmembrane region in the mature protein. Here, we show that the precise architecture of this signal peptide is critical for the correct insertion and maturation of the PsbX protein. PsbX is synthesized with a signal peptide that contains two acidic residues in the C-terminal domain prior to the signal peptidase cleavage site. These negatively charged residues were mutated by IPCR. The resulting mutants were imported *in vitro* into isolated pea chloroplasts. Mutation of these residues to valine does not affect insertion and formation of a loop intermediate, but subsequent maturation is completely inhibited. This is not due to an inability of signal peptidase to cleave at this site; single valine substitutions cause an intermediate block in maturation and the insertion of hydrophilic residues elsewhere in this region rescues processing to the mature size. We propose instead that this region must possess a minimum hydrophilicity in order to be fully translocated into the lumen and made accessible to the signal peptidase. We also provide evidence that these negative charges may actually assist translocation by partially neutralizing positive charges in the translocated region.

44. A Mechanism for Ribosome Exchange on the Endoplasmic Reticulum Membrane

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In current models of protein translocation in the mammalian endoplasmic reticulum (ER), ribosome release from the ER membrane is coupled to the termination of protein translation. In these models, newly released ribosomal subunits enter a free cytoplasmic pool whereupon they can initiate a new round of translation. If engaged in the synthesis of a secretory or membrane protein, the free ribosomes are re-targeted to the ER membrane via the SRP-dependent targeting pathway. In this communication, we report the identification of ribosomes that reside in stable association with the ER membrane following termination of translation. These membrane-bound ribosomes are capable of re-initiating protein translation regardless of whether the mRNA encodes a signal sequence-bearing protein or a soluble, cytoplasmic protein. Membrane-bound ribosomes translating a signal sequence-bearing protein remain bound to the membrane throughout translation and can access the ER translocon in an SRP/SRP receptor-independent manner. In contrast, membrane-bound ribosomes engaged in the synthesis of proteins lacking an exposed signal sequence are released from the ER membrane as intact ribosome/nascent polypeptide complexes. On the basis of these data, we propose that once translation is initiated by a membrane-bound ribosome, continued binding of the translationally active ribosome to the ER is dependent upon direct interactions between the signal sequence and the membrane.

45. Biogenesis of the Amino Terminus of Opsin at the Endoplasmic Reticulum

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Purpose: Secretory proteins contain cleavable signal sequences that target them to the endoplasmic reticulum (ER) membrane for processing. However, many polytopic membrane proteins (those with multiple transmembrane segments) do not have a cleaved amino terminal signal sequence. Instead, the first transmembrane segment serves as the initial signaling information for these proteins. Many of these proteins, including members of the G-protein coupled receptor family (GPCR), also translocate their amino terminus into the lumen of the endoplasmic reticulum (ER), but the mechanism for this translocation is unclear. Our goal is to elucidate the mechanism of this amino-terminal translocation in eukaryotes using protein synthesis intermediates of opsin, a seven transmembrane GPCR. **Methods and Materials:** Opsin constructs truncated at various points along the coding sequence without stop codons were generated by cloning and PCR. These constructs were transcribed *in vitro*. This mRNA was translated *in vitro* under varying conditions. These samples were fractionated using sucrose gradients and analyzed by SDS-PAGE. **Results:** 1) The amino terminus of opsin translocates across the ER as efficiently post-

translationally as it does co-translationally. 2) Efficient translocation of its amino terminus requires the nascent chain to still be attached to its biosynthetic ribosome as well as the addition of GTP and SRP; translocation does not require any additional cytosolic proteins or energy. 3) The amino terminus can translocate post-translationally efficiently even after synthesis of four of the latent transmembrane domains. **Conclusions:** The amino terminus of opsin translocates across the ER in an elongation independent manner with GTP as the only required energy source. This mechanism is distinct from translocation of secretory proteins in mammalian cells, which only occurs co-translationally and requires the signaling information to emerge from the ribosome prior to targeting to the ER.

46. Characterization of the Role of SrrA (GeneX) in the Regulation of the *Escherichia coli* secA Gene

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Our overall goal is to characterize SrrA (formerly GeneX) translation and secretion and its role in secA regulation in *Escherichia coli*. The secA gene is the second gene in the srrA-secA operon and it has been observed that secA translation varies over a tenfold range depending on the status of protein secretion in the cell. secA basal expression is set by a translational coupling mechanism utilizing the upstream gene, srrA. We show by mutational studies and western blots that this secretion-responsive regulation of secA depends on the secretability of SrrA preprotein by the translocon since signal sequence mutations in srrA render secA expression constitutive and they act only when linked in cis to secA. In addition, suppressors of signal sequence defects, prlA (secY) strains containing the srrA signal sequence mutations show partial restoration of secA repression. We also show by genetic studies and by enzymatic assays in srrA-phoA fusion proteins that the GUG codon and not the downstream AUG codon is the correct translation start site of srrA. With GUG as the start codon the newly proposed signal peptide is unusually long for a Gram-negative organism due to its extended N-region (19 residues). Initial characterization of this N-terminal region, which involved substitution of five of the aromatic residues to polar and aliphatic residues show no regulation and high level of basal expression. These results not only confirm that interaction of the srrA preprotein with the translocon is necessary for proper secA regulation but also that the atypical N-terminal region might be playing a unique role in this interaction.

47. A Key Amino Acid is Involved in Regulating SecA-Preprotein Interactions

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SecA is an essential component of the *Escherichia coli* translocon that interacts with SecB, acidic phospholipids, SecY and preproteins. However, the location of SecA's preprotein binding site and the requirements for this interaction remain poorly understood. In order to characterize SecA-preprotein interactions, we conducted a genetic screen on a library of secA mutant alleles. DNA encoding amino

acids 158-421 of SecA was subjected to error prone PCR mutagenesis and cloned into a plasmid containing the geneX-secA operon. The resulting secA alleles were screened for a decrease in their ability to export the product of the malE19-1 gene. Of the 42 mutants chosen for analysis, 36 expressed full length SecA at wild type levels. Only five of these mutants had defects severe enough to show up as export defects in pulse labeling assays. Of these five mutants, only one was able to complement a secA51 (Ts) mutation while being unable to complement a secA13 (Am) mutation. This secA mutant was unable to export preproteins and was unable to generate translocation ATPase activity. This loss of activity was not due to altered localization, YEG binding, ADP binding or ADP release properties, but was due to an altered preprotein binding activity. Endogenous ATPase inhibition assays, fluorescence assays and protease digests revealed that this SecA mutant was able to bind to preproteins with a higher affinity than wild-type SecA and to form more stable SecA-preprotein complexes.

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